



# Using E. coli as an experimental system to study the behavior of prion-like proteins.

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**Using *E. coli* as an experimental system to study the behavior of prion-like proteins**

A dissertation presented

by

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to

The Department of Molecular and Cellular Biology

in partial fulfillment of the requirements

for the degree of

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in the subject of

Biochemistry

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**Using *E. coli* as an experimental system to study the behavior of prion-like proteins.****ABSTRACT**

Prions are infectious, self-propagating protein aggregates that have been uncovered in evolutionary divergent members of the eukaryotic domain of life. It is not known whether prokaryotic organisms contain proteins that exhibit prion-like behavior. However, studies have shown that the *E. coli* cytoplasm can support conversion of the well-characterized *Saccharomyces cerevisiae* yeast prion protein Sup35 into the prion form and that this conversion, like in the yeast system, is dependent on the presence of amyloid aggregates of another yeast prion protein, a so-called PIN factor. It is interesting that the bacterial system recapitulates the *in vivo* requirements for Sup35 prion formation in the native yeast system despite the fact that bacteria diverged from eukaryotes ~2.2 billion years ago. In yeast, once formed, the Sup35 prion is stably propagated and this process is independent of the PIN factor. Using the same yeast prion protein, Sup35, in CHAPTER 2 we show that prion aggregates can be maintained for up to 90 generations in the bacterial cytoplasm and that these aggregates are still infectious when transformed into yeast.

Studies in yeast have implicated cellular chaperones in the *de novo* formation of prion aggregates, but the effect of the bacterial chaperone system on the formation of prion-like aggregates remains to be investigated. In CHAPTER 3, we use the non-prion, amyloid forming bacterial protein CsgB as a model protein in designing an assay to identify cellular factors that interfere with amyloid formation. Using the cytoplasmic amyloid aggregation of this protein as a surrogate for the initial formation of prion-like

aggregates, we identify three open reading frames in *E. coli* that, when overexpressed, potentially inhibit cytoplasmic amyloid aggregation. The work presented in this dissertation demonstrates that *E. coli* can be used as an experimental system to study the behavior of known and putative prion proteins. This system may be especially useful in studying the interaction of prions with cellular chaperones. Our findings increase our interest in the search for bacterial proteins that may be able to function as prions.

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## **CHAPTER 1**

### **Introduction**

## THE PRION PHENOMENON AND MAMMALIAN PRIONS

Prions are infectious, self-propagating protein aggregates that have been uncovered in mammals as well as yeast and other fungi. In mammals, they cause a group of fatal neurodegenerative diseases referred to as the transmissible spongiform encephalopathies (TSEs) [1]. Examples of TSEs that affect humans are Creutzfeldt-Jacob disease (CDJ), Gerstmann-Straussler-Scheinker Syndrome (GSS), fatal familial insomnia (FFI) and Kuru. Well known TSEs that affect other mammals include sheep scrapie and bovine spongiform encephalopathy (BSE), which has been experimentally linked to the novel human prion disease variant vCDJ [2, 3].

Initial studies on the agent responsible for the TSEs revealed that it had unusual characteristics (resistance to high temperatures, chemicals and UV radiation) [3] that did not seem consistent with conventional pathogens such as bacteria or viruses. In 1967, Alper *et al* suggested that the infectious agent of scrapie lacked any nucleic acid [4] and in that same year Griffith formulated the “protein only hypothesis” and proposed three mechanisms to explain the replication mechanism of this infectious agent [5]. Several years later, in 1982 Stanley Prusiner coined the term prion for proteinaceous infectious particle to describe the infectious agent of scrapie in sheep [6].

Since then, work in the field has identified the altered, aggregated form of an endogenous protein called PrP<sup>C</sup> as the causative agent of TSEs [7, 8]. PrP<sup>C</sup> is a cell surface glycosyl phosphatidyl inositol (GPI) linked glycoprotein and it has been identified in all mammals examined to date [9, 10]. Although several functions, including neuroprotective signaling, neurite growth and cell growth and viability in response to oxidative stress, have been proposed for PrP<sup>C</sup>, its physiological role still remains

unknown [11-16]. PrP<sup>C</sup> has an inherent ability to convert to a protease resistant, aggregated form called PrP-scrapie (PrP<sup>Sc</sup>) that is the basis for prion formation. A defining characteristic of these aggregates is that they induce the conversion of normally folded protein to the prion form. PrP<sup>Sc</sup> aggregates are infectious and they can spread not only in infected individuals but also between individuals. Prion disease can be transmitted in laboratory models via intracerebral, intravenous, intraperitoneal and intraocular inoculation of infectious material, but in the field they can also be induced via oral challenge [9].

Infectious PrP aggregates, as well as fungal prion aggregates (see below), are composed of highly structured beta sheet-rich fibrils, known as amyloid fibrils. Within such fibrils the beta-sheets are made up of beta-strands that are oriented perpendicular to the fibril axis, arranged in a “cross-beta structure” [17, 18]. In general, amyloid aggregates are unusually rugged, exhibiting resistance to denaturation in SDS and protease digestion. Amyloid aggregates also characteristically bind the dyes Congo Red and Thioflavin T.

Prion diseases represent just a small subset of a much larger group of protein-misfolding diseases, known as the amyloidoses. There are more than 30 human amyloid diseases (e.g. Alzheimer’s, Huntington’s and Parkinson’s disease) and they are characterized by the deposition of the fibrous aggregates in different tissues. Although the amyloid forming protein in each of these diseases is different, protein misfolding leads in each case to the characteristic cross-beta structure. Contrary to prion diseases, amyloid diseases are typically considered not to be infectious. Interestingly, recent work has raised the possibility that in these diseases the protein misfolding process might

propagate itself in a manner that is mechanistically related to what occurs in prion disease [19-21]. In addition, recent evidence indicated that PrP<sup>C</sup> might play a direct role in the pathogenesis of Alzheimer's disease in particular [22, 23]. Thus, a better understanding of prion diseases and their mechanism of transmission and propagation may shed light into the mechanistic underpinnings of many other devastating and common neurodegenerative disorders.

## **PRIONS IN YEAST**

The discovery of prion forming proteins in fungi and especially yeast cells has led to great advances in the understanding of prion biology. The first yeast prion to be uncovered was the so-called [URE3] prion [24], the prion isoform of the Ure2 protein (Ure2p). (Note that the prion state is designated with capital letters to indicate dominance and brackets to indicate cytoplasmic inheritance.) Ure2p is a regulator of nitrogen catabolism that inhibits the expression of genes required for the uptake of poor nitrogen sources in the presence of a good nitrogen source. However, yeast cells carrying the [URE3] prion lack active Ure2p and as a result take up poor nitrogen sources (such as ureidosuccinate/USA) even though they are grown in the presence of a good nitrogen source [24].

The discovery of the [URE3] prion traces back to 1971, when Francois Lacroute, working on uracil biosynthesis identified mutant yeast cells that could grow on ureidosuccinate despite the presence of ammonium [25]. One of these mutants, named [URE3] was a non-chromosomal mutant and, unlike the other two mutants, displayed a dominant, non-Mendelian fashion of inheritance. Moreover, later studies showed that



chromosomal mutants in *ure2* could not propagate the [URE3] genetic element [26]. Working from these initial observations, in 1994 Reed Wickner presented genetic evidence to suggest that [URE3] was, in fact, a prion [24]. Specifically, the presence of [URE3] resulted in the same loss-of-function phenotype as observed in the case of Ure2p chromosomal mutants [24, 27]. Indeed, in the prion state, the prion protein is sequestered in the prion aggregates and as a result, the prion phenotype typically resembles the loss-of-function phenotype (note that this not true in the case of mammalian prions as deletion of the gene encoding PrP produces no clear phenotype but PrP<sup>sc</sup> is lethal). Additionally, cells cured of [URE3] by treatment with guanidine (see below for a discussion on curing of the prion state), could revert back to the [URE3] state at a frequency similar to the original appearance of [URE3]. Moreover, overproduction of Ure2p, enhanced the frequency of [URE3] appearance by 100-200 fold [27]. Based on these observations, Reed Wicker proposed that [URE3] was a prion of Ure2p. Furthermore, Wickner's observation with [URE3] provided a set of genetic criteria that was used subsequently to identify additional prions in yeast.

It is important to note that, none of the genetic criteria for [URE3] or yeast prions in general apply to mammalian prions [27]. As mentioned above, deletion of the gene encoding PrP does not produce a phenotype similar to the phenotype produced when the PrP protein is in the prion conformation. Also, there are no known cures for TSE and therefore, it is not possible to test the frequency of spontaneous TSE appearance following curing of the prion state. Additionally, overexpression of PrP kills mice, but it does not lead to the generation of the prion state (the tissues of these mice are not infectious) [27]. Finally, yeast prions cause phenotypic changes in yeast cells that are

stably propagated from generation to generation, unlike the prion form of PrP, which causes cell death as it spreads.

Among fungal prions, the Sup35 prion has been the subject of particularly intensive study. Sup35, which is an essential translation termination factor, has a modular structure, with the N terminal region (N) containing the essential prion forming domain (comprising a Q- and N-rich fragment and five copies of an imperfect oligopeptide repeat), and the highly charged middle region (M) enhancing the solubility of Sup35 in the nonprion form and stabilizing the prion form, which is designated  $[PSI^+]$  [28]. The C-terminal domain of Sup35 provides the translation termination activity of Sup35 but is not required for its prion behavior. Studies have shown that, when transferred to heterologous proteins, the N and M regions of Sup35, designated NM, confer prion behavior on the resulting fusion protein [29].

In  $[psi^-]$  cells (i.e. cells lacking the Sup35 prion), Sup35 is soluble and able to properly terminate translation. In  $[PSI^+]$  cells, however, Sup35 is largely sequestered in prion aggregates, resulting in impaired translation termination and the suppression of nonsense mutations. The  $[PSI^+]$  state can easily be detected by suppression of nonsense-codon mutations in auxotrophic markers [30]. The  $[PSI^+]$  state, like prion states in general, is inherited in a dominant, non-Mendelian fashion and is stably propagated over multiple generations.

The spontaneous conversion of the Sup35 from the soluble form to the prion form is a rare event with a frequency of  $10^{-6}$  per total colonies analyzed, and is dependent on the presence of yet another yeast prion referred to as PIN, for  $[PSI^+]$  inducibility, factor [31]. PIN is required for the *de novo* appearance of  $[PSI^+]$ , but not for its propagation [32, 33].

The prion-forming properties of Ure2p and Sup35 depend on a glutamine and asparagine rich (Q/N-rich) region within the N terminal domain of each protein. Genome wide searches for Q/N-rich domains have led to the identification of other yeast proteins with prion domains, including New1p and Rnq1p. (Note that the Q/N-rich domain of New1 is also referred to as the NYN repeat region.) Although no phenotypic changes have yet been associated with the inactivation of these two proteins, in its prion form, each one functions as a PIN factor, rendering the cells [*PIN*<sup>+</sup>] [32, 34, 35]

As already mentioned, an important difference between mammalian and yeast prions is that, unlike mammalian prions, yeast and fungal prions do not typically result in cell death. In fact, recent evidence suggests that prion formation may be beneficial under particular circumstances [36-38] but see [39, 40], for the opposing view. A growing body of work suggests that prions do not represent disease states in yeast, but instead that conversion to the prion state might serve as mechanism to reversibly modulate the phenotype in fluctuating environments. Researchers have argued that conversion to the prion state could allow yeast cells to test normally suppressed phenotypes and could promote adaptive evolvability [36]. Consistent with this hypothesis, studies have shown that stress states such as oxidative stress or high salt concentrations can increase the conversion frequency of Sup35 to the prion state [41]. The known repertoire of prions in the fungal kingdom is rapidly expanding, providing further support for the idea that heritable protein aggregates have functional and beneficial roles in the cell. Indeed, yeast prion proteins have been shown to play many different cellular roles including but not limited to, chromatin remodeling [42, 43], transcriptional regulation [42, 44] and cell cycle dynamics [42, 45].

For example, [*PSI*<sup>+</sup>] strains of *Saccharomyces cerevisiae* were found to exhibit enhanced tolerance to heat and chemical stress as compared to [*psi*<sup>-</sup>] yeast strains [37, 46, 47]. However, if prions are beneficial, then they should be found in wild strains as well as laboratory strains. This was the motivation for the study by Linquist and colleagues, which looked beyond Sup35 to ask about prions more generally. In this study, the authors tested wild strain of *Saccharomyces* for presence of prions and found them in a considerable fraction of the strains tested [38]. The group also found that these prion states conferred phenotypes that were beneficial under some stressful conditions tested.

Other groups have also identified yeast prions that result in phenotypic changes that prove advantageous under certain environmental stress conditions. For example, in its prion state, the recently identified yeast prion protein Mod5 confers cellular resistance against antifungal agents and furthermore, selective pressure by antifungal drugs increases the *de novo* appearance of the Mod5 prion state (designated [*MOD*<sup>+</sup>]) in yeast [48]. Interestingly, unlike other yeast prion proteins, Mod5 does not contain a Q/N-rich region [44, 48].

Aside from the prions described in yeast, another prion was shown to control heterokaryon incompatibility in the evolutionary divergent filamentous fungus, *Podospora anserina* [17]. Filamentous fungi have developed self/non-self recognition systems limiting the cellular fusion of different colonies to genetically identical partners. This process is dependent on the *het*-loci and *P. anserina* has nine such loci, one of which, *het-s*, involves a prion protein [49]. The *het-s* locus has two allelic variants named *het-s* and *het-S*. Strains expressing the Het-s protein in the soluble form are compatible with strain expressing the Het-S protein and these strains can engage in cellular fusion.

However, the Het-s protein can spontaneously convert to the prion conformation (termed [Het-s], making the strains harboring the prion incompatible with the strains harboring the Het-S protein [50]. A fusion event between these two strains results in cell death (heterokaryon incompatibility). A cell with the [Het-s] phenotype can however fuse to a cell expressing the Het-s protein and, when this happens, the [Het-s] amyloid spreads to the other cell and converts it to the [Het-s] compatibility phenotype [49]. Due to its involvement in heterokaryon incompatibility, the prion form of Het-s represents another example of prions having functional roles within the cell.

### **Role of the chaperone systems in yeast prion propagation**

Propagation of prions depends on their ability to grow the aggregate by recruiting soluble protein of the same type and converting it to the amyloid form. However, the amyloid fibers need also to be fragmented in order to create prion seeds that function as new catalytic ends to recruit additional soluble protein. This process of propagation and fiber fragmentation in mammals is still not understood.

Studies in yeast however, have implicated cellular chaperones and the heat-shock protein Hsp104, in particular, in the propagation of yeast prions. Hsp104 is a member of the Hsp100/ClpB family of the hexameric AAA<sup>+</sup> ATPases. This family of proteins includes bacterial, fungal and plant ATPases [51-54]. The Hsp104/ClpB chaperones work together with the Hsp70/40 chaperone system to break down large protein aggregates or remodel non-natively folded polypeptides [55-57]. This process involves the extraction of single polypeptide chains from the aggregates and threading them through the central channel of the Hsp100/ClpB hexamer [58, 59].

Hsp104 was initially identified as an important factor for the development of thermotolerance in yeast [60, 61]. Studies have shown that, under stress conditions, yeast strains missing or containing a mutated Hsp104 have a reduced survival rate as compared to wild-type strains [62, 63]. Under normal growth conditions, Hsp104 plays a critical role in prion propagation. Thus, cells lacking Hsp104 are unable to propagate any of the known yeast prions, including [*PSI*<sup>+</sup>]. Moreover, the inhibition of Hsp104 activity, which can be accomplished by growing cells in the presence of moderate concentrations of guanidine hydrochloride (GdnHCl), cures cells of most, although not all, prions. Various lines of evidence suggest that the essential role of Hsp104 in prion propagation is to break apart large aggregates, generating smaller seeding-competent particles that can more effectively be partitioned to daughter cells [64-68], but the exact role of Hsp104 in the prion process remains controversial [69, 70]. In the case of [*PSI*<sup>+</sup>], but not for other yeast prions such as [*URE3*] or [*PIN*<sup>+</sup>], Hsp104 overproduction also cures cells of the prion; the mechanism by which this occurs is not fully understood [42].

In addition to Hsp104, many other yeast chaperones have been implicated in prion biogenesis and propagation, though their effects are complex and have been difficult to disentangle. Hsp70s are essential, universally conserved chaperones and in yeast, along with their obligatory Hsp40 partners, they are necessary for the role of Hsp104 in solubilizing protein aggregates. Essential for the activity of Hsp70 are the nucleotide exchange factors (NEFs). Yeast contain 6 different Hsp70 chaperone proteins, 2 major nucleotide exchange factors and over a dozen Hsp40 chaperone proteins [71, 72]. Interestingly, the Hsp70/Ssa1 and Hsp40/Sis1 chaperones appear to influence the propagation of most yeast prions [73-77]. However, their exact involvement and role in

yeast prion propagation has been very difficult to elucidate as some chaperones promote aggregation of one prion while eliminating aggregation of another and sometimes the same chaperone has opposing effects even on the same yeast prion depending on the chaperone environment of the cell (presence or absence of other chaperones) [77, 78]. Furthermore, it has been shown that even the nucleotide exchange factors (NEFs) of Hsp70 can also influence prion aggregates in yeast [79, 80]. Although the effect of the chaperone systems on yeast prions is very complex and not yet fully understood, it is clear that chaperones play a crucial role in the maintenance and propagation of yeast prions and therefore are intricately linked to prion biology.

## **PRION STRAIN AND SPECIES BARRIERS**

Although all mammalian prion diseases (TSEs) are mainly characterized by neuronal cell loss in the central nervous system and deposition of amyloid plaques, they are very variable in their clinical presentation and neuropathological pattern. These differences are believed to be due to differences in the PrP<sup>Sc</sup> tertiary structure, and the different conformations are considered to be different prion strains. Indeed, the existence of prion strains was observed as early as 1961 by Pattison and Millson [81], who found that goats infected with the same batch of scrapie agent developed different clinical phenotypes. Subsequently, work from a number of different groups has indicated that the same protein, PrP<sup>C</sup>, can adopt different, stable PrP<sup>Sc</sup> conformations, that in turn define different disease phenotypes [82-85].

Prion strains have also been observed in the case of yeast prions. The best characterized examples involve the *S. cerevisiae* prion protein Sup35. Sup35 is a

translation release factor and, in its normal, soluble, non-prion conformation, Sup35 is free to carry out its role in efficiently terminating translation in the cytoplasm. In its prion form, Sup35 is sequestered in amyloid aggregates and, as a consequence it has a reduced ability to efficiently terminate translation resulting in a nonsense suppression phenotype referred to as  $[PSI^+]$  [30]. The suppression of nonsense-codon mutations in auxotrophic markers is commonly used in yeast to detect the  $[PSI^+]$  state. More specifically, in yeast containing a nonsense mutation in the *ade1* gene,  $[PSI^+]$  colonies are white or pink (and can grow in the absence of adenine), whereas  $[psi^-]$  colonies are red (and require adenine to grow). Studies have shown that different  $[PSI^+]$  strains can arise in genetically identical yeast cells [86]. Along with differences in their mitotic stability and dependence on the cellular chaperone machinery, the  $[PSI^+]$  strains differ in their translation termination defects, which in turn can be visualized as differences in the *ade1* color phenotype [30, 87].

Additionally, there is evidence to suggest that conformational differences in the Sup35 aggregates underlie the different levels of heritable nonsense suppression in different  $[PSI^+]$  strains [86, 88]. Using Sup35 aggregates formed *in vitro* under different conditions, Tanaka *et al.* showed that the different polymerization conditions resulted in distinct Sup35 aggregates, which, when transferred into  $[psi^-]$  yeast by protein transformation (experimental infection), conferred differing nonsense suppression phenotypes on the recipient cells [87]. In these experiments, the Sup35 aggregates formed at 4°C, had different physical properties than the aggregates formed at 23°C or 37°C, and they had a high efficiency of infection with the majority of the colonies showing the strong (white)  $[PSI^+]$  phenotype [87]. The Sup35 aggregates formed at 23°C or 37°C had



instead, low infectivity with almost all the colonies showing the weak (pink) [*PSI*<sup>+</sup>] phenotype [87]. Additional studies by Diaz-Avalos *et al*, provided further evidence that conformational differences in Sup35 amyloid underlie the differences in the heritable nonsense suppression phenotypes of different [*PSI*<sup>+</sup>] strains [89].

The concept of prion strains has also been used to explain the observation that prion infections are typically restricted by species barriers; prion disease transmission is much more efficient within the same species than between different species. For example, due to a very strong species barrier, there hasn't been a recorded case of sheep to human scrapie transmission. Although PrP<sup>C</sup> is highly conserved between species, there are a few polymorphic positions where different species have different amino acid residues. These amino acid differences influence the misfolding propensity of PrP<sup>C</sup>, as well as the details of the misfolded conformation. When a prion strain of one species infects an animal of a different species, if the PrP<sup>Sc</sup> agent represents a different misfolded conformation than the one favored by the soluble PrP<sup>C</sup> of the host, the soluble, host PrP<sup>C</sup> will not convert into the prion form and there will be a species barrier to transmission. Nonetheless, in certain cases prion strains arise that can breach a species barrier. An example is seen in the human prion disease variant vCDJ, which has been experimentally linked to the consumption of meat obtained from cattle infected with bovine spongiform encephalopathy [90, 91].

Barriers to transmission have been described in yeast as well. Using a genetic system designed to mimic infection experiments performed with mammalian prions, studies from the Weissman laboratory [92] elegantly demonstrated that, although capable of forming heritable conformations independently, the prion domains of Sup35 from *Saccharomyces cerevisiae* and *Candida albicans* are incapable of cross seeding each other. However,

studies from the same lab showed that, a hybrid *S. cerevisiae*/ *C. albicans* Sup35 protein could “infect” both organisms [93]. Moreover, these workers showed that different amino acid substitutions in this hybrid protein affected the tendency of the protein to adopt distinct amyloid conformations, which in turn affected the ability of the aggregates to “infect” one organism or the other [94]. These experiments served as a model to better understand the nature of species barriers and provided further support for the idea that conformational differences are the primary determinant of barriers to transmission between different species.

## **BACTERIAL PRIONS?**

Although work conducted over the last decade has uncovered putative prion proteins in evolutionarily divergent members of the fungal Kingdom, it is not yet known how widespread prions are in nature and more specifically if bacteria contain prions. If such proteins exist, bacteria could utilize prion-like aggregates as epigenetic switches that could potentially help the cells adapt to a variety of stress conditions. Indeed, a number of mechanisms, including phase variation systems and bistable switches, have been described that enable isogenic populations of bacteria to exhibit phenotypic diversity. These systems are thought to increase the chance of cell survival under adverse conditions [95-97]. These processes are reminiscent of the role yeast prions play in generating phenotypic diversity in yeast. It is possible that prion formation in bacteria may provide another yet-to-be uncovered mechanism for generating phenotypic diversity.

Although prions have not yet been described in bacteria, non-prion amyloid forming proteins have been discovered in many bacterial species. In particular, many bacteria,

including *E. coli*, elaborate surface-associated amyloid fibrils that are made up of amyloidogenic proteins that are exported to the cell surface where they adopt the amyloid fold. These cell surface-associated amyloid fibrils contribute to the formation of biofilms, which consist of aggregates of cells embedded in an extracellular matrix consisting of polysaccharides, proteins, nucleic acids and lipids useful for surface adhesion and colony formation [97, 98]. Because these amyloids play an important role in the formation of biofilms, they are referred to as functional amyloids. This term is used to distinguish amyloid fibers with defined biological functions from those associated with pathological processes.

In *E. coli*, these cell surface-associated fibers are known as curli fibers. The curli fibers are comprised of the major subunit CsgA and the minor subunit CsgB. Both of these proteins have signal sequences to direct their export to the cell surface. In addition, CsgB has a C-terminal sequence that mediates its attachment to the outer membrane and from there it templates the amyloid polymerization of CsgA to form the curli fiber. Studies have shown that CsgB can form cytoplasmic amyloid if its N-terminal signal sequence is removed [99-102].

### **Yeast prions can access the infectious conformation in *E. coli***

While it is not known if bacteria contain prions, whether or not the bacterial cytoplasm provides a suitable environment for the formation of prions has been tested. A study from this lab has shown that *E. coli* provides an environment suitable for the conversion of the yeast prion Sup35NM domain into the prion form. This study showed that the Sup35NM moiety can access an infectious prion conformation in *E. coli* cells,

and this conversion is dependent on a transplanted [*PIN*<sup>+</sup>] factor, the prion form of New1p [103]. Specifically, the transfer of cellular protein from the *E. coli* cells containing both Sup35NM and New1 aggregates to [*pin*<sup>-</sup>][*psi*<sup>-</sup>] yeast cells resulted in [*PSI*<sup>+</sup>] convertants, demonstrating that the Sup35 aggregates generated in *E. coli* cells are infectious and therefore satisfy the essential criterion that defines a prion. It is striking that the conversion of Sup35NM to the prion form in the bacterial cells, like in yeast cells, is dependent on the presence of a [*PIN*<sup>+</sup>] factor, especially considering the fact that bacteria diverged from eukaryotes ~2.2 billion years ago.

## FOCUS OF THE DISSERTATION

The work presented in this dissertation is focused on using *E. coli* cells as a new experimental system for studying prions and their interactions with cellular chaperone proteins. The specific focus of this work has been to (i) study the heritability of yeast prions in the bacterial environment as well as (ii) identify bacterial chaperones that influence amyloid aggregation and potentially prion formation.

Whereas studies in this lab had already shown that Sup35NM could access the infectious prion conformation in the *E. coli* cytoplasm, we wanted to investigate the ability of these prions to be maintained and stably inherited in the *E. coli*. Using the well-characterized *Saccharomyces cerevisiae* yeast prion protein Sup35, we have shown that prion aggregates can be maintained for up to 90 generations in the bacterial cytoplasm (CHAPTER 2) and that these aggregates are still infectious when transformed into [*psi*<sup>-</sup>] yeast cells.

By showing that the bacterial cytoplasm can support the formation and also

propagation of infectious prion aggregates, our lab has shown that bacterial cells can provide a heterologous system in which to study prion biology. Bacteria encode homologs of the chaperones implicated in prion propagation in yeast while at the same time containing a reduced number of chaperones compared to yeast [71, 104-106]. For these reasons, we believe that *E. coli* cells provide a simpler system to study the complex interaction of prion proteins with cellular chaperones. It is striking that the conversion of Sup35<sup>NM</sup> to the prion form in the bacterial cells, like in yeast cells, is dependent on the presence of a [*PIN*<sup>+</sup>] factor. Based on these similarities, we were interested in exploring the effect of bacterial chaperone systems on the formation of prion-like aggregates. In fact, studies in yeast have implicated cellular chaperones in the *de novo* formation of [*PSI*<sup>+</sup>] [107, 108]. Using the cytoplasmic amyloid aggregation of the curli component CsgB as a surrogate for the initial formation of prion-like aggregates, we identified three open reading frames in *E. coli* that, when overexpressed, potentially inhibit cytoplasmic amyloid aggregation (CHAPTER 3).

The work presented in this dissertation demonstrates that *E. coli* can be used as an experimental system to study the behavior of known and putative prion proteins. The system we use to study prion heritability in the *E. coli* cytoplasm can also be used to test the effect specific bacterial chaperones have on this process. The demonstration that the bacterial environment supports the formation and propagation of the Sup35 prion increases our interest in searching for bacterial proteins that may similarly be able to function as prions.

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## **CHAPTER 2**

### **Propagation of a yeast prion protein in *E. coli***

## **ATTRIBUTIONS**

I performed the experiments presented in this chapter. Critical advice, reagents and directions on methods were provided by Dr. Sean J. Garrity.

## ABSTRACT

Prions are infectious, self-propagating protein aggregates that have been uncovered in evolutionary divergent members of the eukaryotic domain of life. However, it is not yet known how widespread prions are in nature and, more specifically, if bacteria contain prions or even if their cytoplasm provides a suitable environment for prion formation and propagation. Here we demonstrate that the *E. coli* environment can support the maintenance of the prion form of a well-characterized yeast prion protein, Sup35. We also demonstrate that, like in yeast cells, this process is not dependent on the presence of a PIN factor, a distinct prion required for the *de novo* appearance of the Sup35 prion in yeast, but not for its subsequent propagation. Our results indicate that the bacterial cytoplasm can be used as a simpler system in which to study the interaction of chaperones with yeast prion proteins.



## INTRODUCTION

Prions are infectious, self-propagating protein aggregates that have been associated with a number of devastating diseases. Prion aggregates have a characteristic amyloid structure, consisting of beta-sheet-rich fibrils, where the beta- strands run perpendicular to the fibril axis [1, 2]. In mammals, prions cause fatal neurodegenerative diseases referred to as transmissible spongiform encephalopathies (TSEs)[3].

The discovery of prion forming proteins in fungi and especially yeast cells has led to great advances in the understanding of prion biology. Unlike mammalian prions, yeast prions do not result in cell death and have even been shown to have physiological functions. Furthermore, because they are self-propagating, yeast prions act as protein-based hereditary elements that are stably maintained from generation to generation.

The stable maintenance of yeast prions is critically dependent on the cellular chaperone network [4, 5]. In particular, studies in yeast have suggested a critical role for the Hsp104 chaperone in prion heritability. Hsp104 is a member of the Hsp100 AAA+ family of ATPases and, in conjunction with the yeast Hsp70/Hsp40 system, it functions to disaggregate and reactivate misfolded proteins in the cell [6, 7]. Evidence suggests that the essential role of Hsp104 in prion propagation is to sever large prion aggregates into multiple, smaller aggregates that can act as seeds for additional rounds of polymerization and can more easily be propagated to daughter cells. However, the exact involvement of Hsp104 in the prion process remains controversial [8, 9]. In addition to Hsp104, several other yeast chaperones, including the Hsp70/Hsp40 chaperone system, have been implicated in prion propagation, although their effects are complex and not well understood.

A particularly well-characterized prion in *Saccharomyces cerevisiae*, the  $[PSI^+]$  prion, is formed by the essential translation termination factor Sup35, which assembles into amyloid aggregates when it converts into the prion form. Sup35 has a modular structure, with the N terminal region containing the essential prion forming domain, the highly charged middle region (M) enhancing the solubility of Sup35 in the non-prion form and stabilizing the prion form  $[PSI^+]$ , and the C-terminal domain comprising the translation termination activity of Sup35 (but not required for its prion behavior) [10]. Studies have shown that, when transferred to heterologous proteins, the N and M fragments of Sup35, designated NM, confer prion behavior on the resulting fusion proteins [11]. In yeast, the *de novo* appearance of  $[PSI^+]$  (but not its propagation) is dependent on the prion form of yet another prion protein called a  $[PIN^+]$ , for  $[PSI^+]$  inducibility, factor [12-15]. In yeast the New1 and Rnq1 proteins both function as  $[PIN^+]$  factors.

In  $[psi^-]$  cells, Sup35 is soluble and able to properly terminate translation. In  $[PSI^+]$  cells, however, Sup35 is largely sequestered in prion aggregates, resulting in impaired translation termination and the suppression of nonsense mutations. The  $[PSI^+]$  state can easily be detected by suppression of nonsense-codon mutations in auxotrophic markers [16]. In yeast containing a nonsense mutation in the *ade1* gene,  $[PSI^+]$  colonies are white (referred to as strong  $[PSI^+]$ ) or pink (referred to as weak  $[PSI^+]$ ), whereas  $[psi^-]$  colonies are red.

Although work conducted over the last decade has uncovered putative prion proteins in evolutionary divergent members of the fungal Kingdom, it is not yet known how widespread prions are in nature and, more specifically, if bacteria contain prions or even if their cytoplasm provides a suitable environment for prion propagation. A study from

this lab has shown that *E. coli* can indeed provide an environment suitable for the conversion of the yeast Sup35NM domain into the prion form [17], and that conversion of the Sup35NM moiety into the prion conformation is dependent on a transplanted [*PIN*<sup>+</sup>] factor, the prion form of New1 [17]. Specifically, *E. coli* cells producing both Sup35NM and New1 (but not Sup35NM alone) contained material that could induce [*PSI*<sup>+</sup>] when transferred to yeast cells containing Sup35 in the non-prion form, demonstrating that the Sup35NM aggregates generated in *E. coli* cells are infectious and therefore satisfy the essential criterion that defines a prion. However, the ability of the infectious Sup35NM aggregates to be propagated and stably maintained in the bacterial cytoplasm remains to be investigated.

Here we show that the Sup35NM prion formed in the bacterial cytoplasm in the presence of the transplanted PIN factor, New1, can be maintained in the bacterial cytoplasm for close to 90 generations following removal of the PIN factor. These results suggest that the bacterial cytoplasm can be used as a heterologous system to study the complex interaction of prions and chaperone proteins.

## RESULTS

**The infectious Sup35 prion aggregates can be maintained in the bacterial cytoplasm for 30 generations following the shut off of PIN synthesis.**

Whereas studies in this lab have already shown that Sup35NM can access the infectious prion conformation in the *E. coli* cytoplasm, we sought to determine whether these prions could be maintained and stably inherited in the *E. coli* environment. To address the question of the heritability of the Sup35NM (hereafter referred to as NM) aggregates in the bacterial environment, we took advantage of the fact that, in yeast, the  $[PIN^+]$  factor is required for the *de novo* appearance of  $[PSI^+]$ , but not for its propagation. Our plan was to induce the formation of NM aggregates in *E. coli* cells also containing the New1 prion domain serving as the PIN factor, and then to monitor the propagation of the NM aggregates after New1 synthesis was repressed.

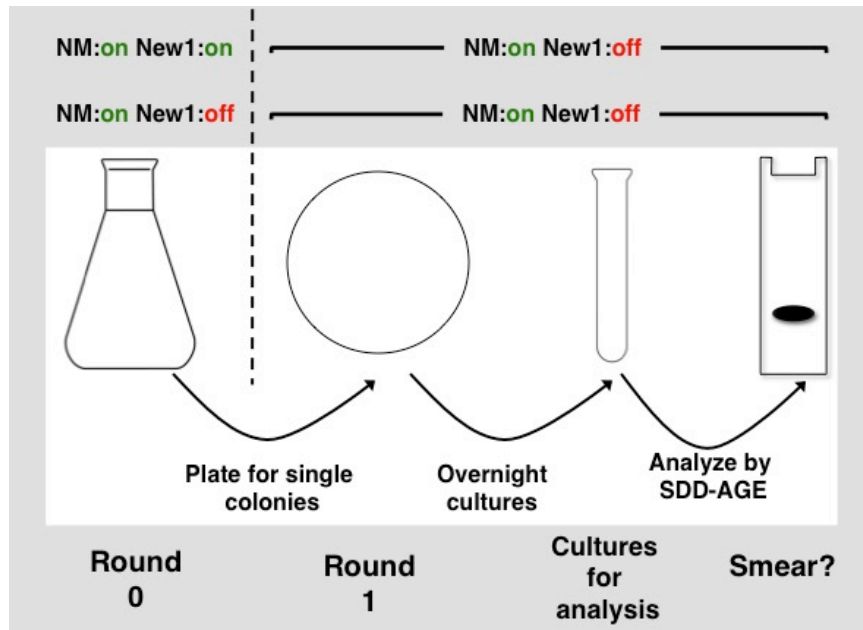
To carry out this experiment, we made use of a plasmid vector directing the arabinose-inducible synthesis of an NM-YFP fusion protein and an *E. coli* strain modified to direct the IPTG-inducible synthesis of a New1-CFP fusion protein from the chromosome. We grew cells containing the NM-YFP plasmid for about 5 hours in liquid culture under inducing conditions for both NM and New1 (in the presence of both arabinose and IPTG- hereafter this culture will be referred to as culture A), thus allowing the NM aggregates to form. We also grew a control culture containing arabinose but no IPTG (hereafter referred to as culture B).

Following formation of the NM aggregates in culture A, we plated the cells from both cultures on medium containing arabinose, but not IPTG, to allow for continued synthesis of only the NM-YFP fusion protein (New1-CFP synthesis was shut off). Individual

colonies were picked and inoculated into liquid medium for overnight growth. Cell extracts were prepared from these overnight cultures and analyzed for the presence of NM aggregates by means of semidenaturing detergent agarose gel electrophoresis (SDD-AGE), a technique that permits the visualization of SDS-stable amyloid polymers. For this analysis, bacterial extracts are treated at room temperature with 2% SDS and then run through a 1.5% agarose gel containing 0.1% SDS that allows for separation of SDS-stable polymers from soluble protein. With this method, the SDS-stable polymers are visualized as higher molecular weight smears. The experimental approach is shown in Figure 2.1.

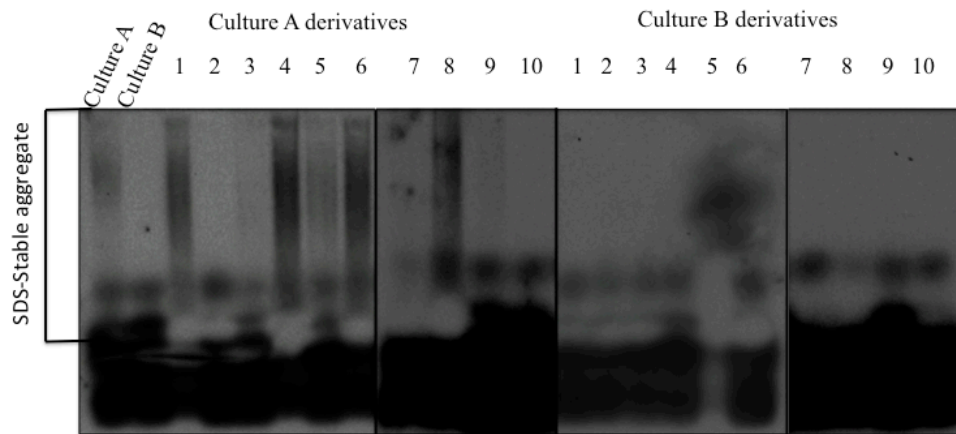
In this experiment, we examined 10 samples derived from the starter culture induced for both NM and New1 synthesis (culture A) and 10 samples derived from the starter culture induced for NM only (culture B). Of the 10 samples derived from Culture A, eight contained NM aggregates (Nr.1, 3, 4, 5, 6, 7, 8, 9) (Fig. 2.2A). None of the cultures derived from the Culture B contained detectable NM aggregates. These same samples were subjected to Western blot analysis in order to assess fusion protein levels (Figure 2.2B and 2.2C).

This initial experiment shows that NM aggregates can be maintained for up to 30 generations (~20 generations on solid medium plus ~10 generations during overnight growth in liquid medium) following the shut off of New1 synthesis. Although there are low levels of New1 protein in culture A derivatives due to leaky *new1* expression (Fig. 2.2C), these protein levels are not sufficient to lead to *de novo* NM aggregation (S. J. Garrity, unpublished data). In this experiment, we observed low levels of New1 protein only in culture A derivatives, but not in culture B derivatives. However, in a repeat

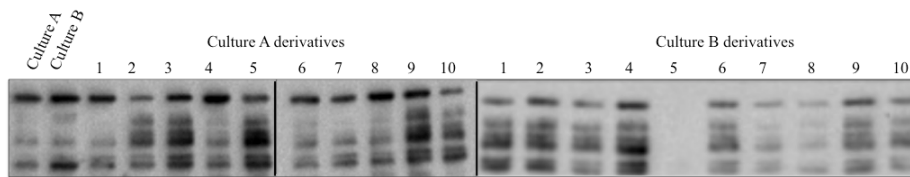


**Figure 2.1: Experimental design for studying prion heritability in *E. coli* following shut off of New1 synthesis.** NM aggregates are allowed to form in the bacterial cytoplasm in the presence of the New1-CFP fusion protein in Round 0. Cells are then grown for 30 generations under conditions that repress New1-CFP synthesis, and then analyzed for NM SDS-stable aggregates by SDD-AGE.

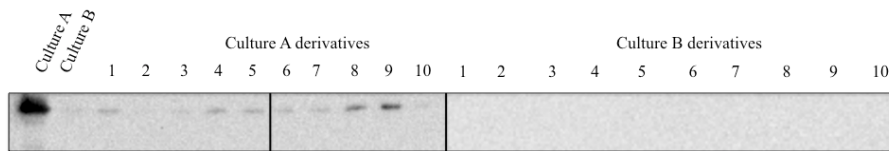
A.



B.



C.



**Figure 2.2: NM aggregates can be maintained for 30 generations in *E. coli* in the absence of New1.** (A) SDD-AGE analysis of cultures from Round 1 of experimental design shown in Figure 2.1. This gel image is a composite of separate gels. Lanes 1 and 2 contain the cultures A and B respectively. The next 10 lanes contain extracts from overnight cultures grown from 10 separate Round 1 colonies derived from culture A. Eight of these samples (Nr.1, 3, 4, 5, 6, 7, 8, 9) still contained NM aggregates although *new1* expression had been shut off for 30 generation. The last 10 lanes contain extracts from overnight cultures grown from 10 separate Round 1 colonies derived from culture B. None of these samples contained detectable NM aggregates. We frequently observe signal-producing shapes in the SDD-AGE blots that are not to be confused with higher molecular weight aggregates (see signal between lanes 5 and 6 in culture B derivatives). We are able to distinguish these signals from real smears corresponding to NM aggregates as smears typically follow the contours of the lanes and have distinct edges. The blot was probed with an anti-Sup35NM antibody.

(B) Western analysis of NM protein levels of the samples in (A). As seen from the blot, for reasons we do not understand, the NM protein levels vary significantly in the steps following Round 0. The blot was probed with an anti-Sup35NM antibody. The top band

### Figure 2.2 continued

corresponds to the NM-YFP fusion protein. The bands observed below the full-length fusion protein represent degradation products. (C) Western blot analysis of New1 protein levels of the samples in (A). The blot was probed with anti-HA antibody as the New1 protein is HA tagged. We still observed residual New1 levels in a subset of the cultures where New1 synthesis had been shut off for 30 generations, which we attribute to leaky expression of the *new1* gene.



experiment, low levels of New1 protein were observed in samples from both culture A and B derivatives (unpublished data).

We observed considerable variability in the levels of full-length NM-YFP protein between samples from either culture A or B derivatives (Fig 2.2B). We do not fully understand the reason for the observed variability in the levels of full-length NM protein, which has hampered our ability to study the heritability of the NM aggregates. In particular, as the maintenance of prion-like aggregates necessarily depends on the uninterrupted synthesis of the corresponding prion protein, our current experimental set-up does not allow us to draw any conclusion about the stability of the NM prion in the *E. coli* system (see SUPPLEMENT TO CHAPTER 2 for a more detailed analysis of variability in the levels of full-length NM protein).

To determine whether the NM aggregates observed in culture A derivatives represent infectious material, we used the material to transform  $[pin^-][psi^-]$  yeast cells and asked whether any of the transformed cells were converted to  $[PSI^+]$ . To carry out this experiment, we prepared cell extracts from both starter cultures (+ and – IPTG) to serve as positive and negative controls, respectively, as well as from the culture A derivatives Nr.4 and Nr.5. We mixed the bacterial cell extracts with a plasmid DNA encoding a yeast selectable marker and used them to transform yeast spheroplasts prepared from a  $[pin^-][psi^-]$  yeast strain. The choice of a  $[pin^-][psi^-]$  yeast strain was crucial as transient overproduction of NM in a  $[PIN^+][psi^-]$  yeast strain stimulates conversion to  $[PSI^+]$  [18].

Following the transformation, we looked for possible  $[PSI^+]$  transformants among the total transformants, which are visible as white colonies on ¼ YPD plates. Candidate  $[PSI^+]$  clones were passaged on guanidine hydrochloride-containing medium and those

that were cured (reverted to  $[psi^-]$ ) were scored as  $[PSI^+]$ . Our analysis showed that the extracts from culture A derivatives Nr.4 and Nr.5, samples containing heritable aggregates, resulted in 2.39% and 0.4%  $[PSI^+]$  yeast transformants, respectively, as compared to 1.56%  $[PSI^+]$  yeast transformants in the case of the positive control extract from culture A (see Table 2.1). Interestingly, all of the  $[PSI^+]$  yeast transformants observed in this experiment were strong  $[PSI^+]$  strains [19]. The extract from the starter culture grown in the absence of IPTG did not result in any  $[PSI^+]$  yeast transformants, which is consistent with the absence of NM aggregates in this sample. The observed conversion frequencies observed in this experiment are consistent with conversion frequencies from previous experiments in this lab [17]. These results clearly show that infectious NM aggregates can be maintained in the bacterial cytoplasm for up to 30 generations following the shut off of New1 synthesis.

### **Heat shock does not have a negative impact on the heritability of the infectious NM prion aggregates in the bacterial cytoplasm.**

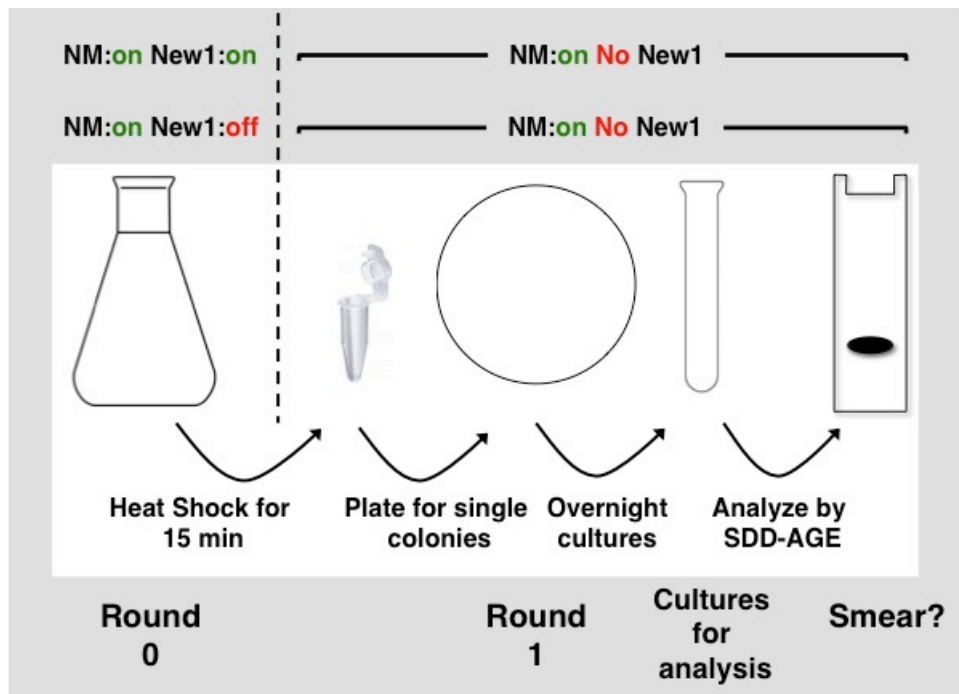
Because we can observe low New1 levels even in bacterial cultures grown in the absence of IPTG due to the leaky *new1* expression, we wished to exclude the possibility that a low level of New1 synthesis might be required to maintain the NM prion. The plan was to induce the formation of NM aggregates in *E. coli* cells containing the New1 prion domain, and then monitor the propagation of the NM aggregates after the *new1* gene was removed from the system. To carry out this experiment, we took advantage of the fact that the *new1* gene is inserted in the *E. coli* genome at the lambda attachment site (attB),

**Table 2.1: NM aggregates maintained in *E. coli* in the absence of New1 for 30 generations are infectious when transformed into yeast cells.** Extracts from bacterial cultures shown in the table above were used to transform [*pin*<sup>-</sup>][*psi*<sup>-</sup>] yeast cells. The [*psi*<sup>-</sup>] to [*PSI*<sup>+</sup>] conversion percentages for the yeast transformants are shown. Analysis of these data by Fisher's exact test suggests that the observed difference in the frequencies of [*PSI*<sup>+</sup>] transformants is statistically significant ( $P < 10^{-3}$ ).

Bacterial Extract	% of [ <i>PSI</i> <sup>+</sup> ] Transformants
Culture A	1.56% (58/3656)
Culture B	0% (0/2932)
Culture A derivative Nr.4	2.39% (93/3892)
Culture A derivative Nr.5	0.4% (13/3276)

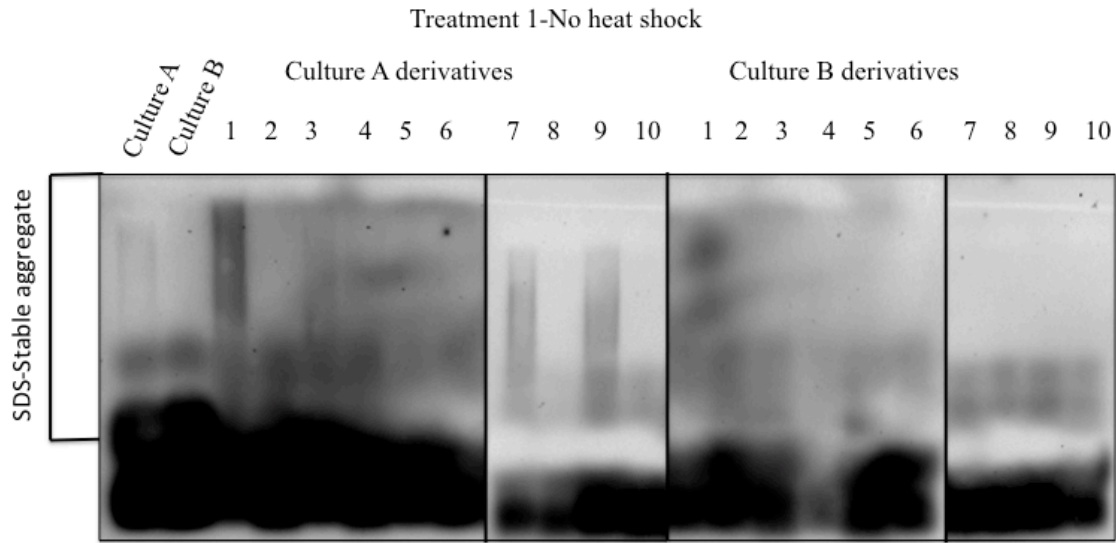
enabling us to excise the *newI* gene from the chromosome after the initial formation of the NM aggregates by providing the enzymes required for the excision reaction (the bacteriophage lambda Int and Xis proteins) *in trans* on a temperature-inducible plasmid [20]. Subjecting the bacterial cells carrying this plasmid to a 42<sup>0</sup>C heat shock treatment, induces expression of the excision enzymes, which in turn leads to removal of *newI* from the bacterial chromosome (see Figure 2.3 for modified experimental approach).

Since the new experimental approach includes a heat shock step, a process known to affect protein folding, we first decided to test the effect of this added step on the heritability of the NM aggregates. It is important point out that in this preliminary experiment the bacterial cells did not carry the plasmid encoding the excision machinery and therefore, the *newI* gene was not excised. To carry out this preliminary experiment, following formation of NM aggregates, we treated two equal aliquots from each of the cultures A and B in two different ways. One pair was plated directly on medium containing arabinose, but not IPTG, to allow for continued synthesis of only the NM-YFP fusion protein but not New1-CFP (Treatment 1). The other pair of samples was subjected to a 15-minute heat shock step before being plated on the same medium (Treatment 2). For each treatment, we then picked 10 individual colonies derived from each of the starter cultures and analyzed their extracts for presence of NM aggregates. In the case where cultures A and B were subjected to treatment 1 (Fig. 2.4A), of the 10 samples derived from culture A, 5 contained NM aggregates (Nr.1, 3, 4, 7 and 9), while none of the cultures derived from culture B contained detectable NM aggregates. In the case where cultures A and B were subjected to treatment 2 (Fig. 2.4B), of the 10 samples

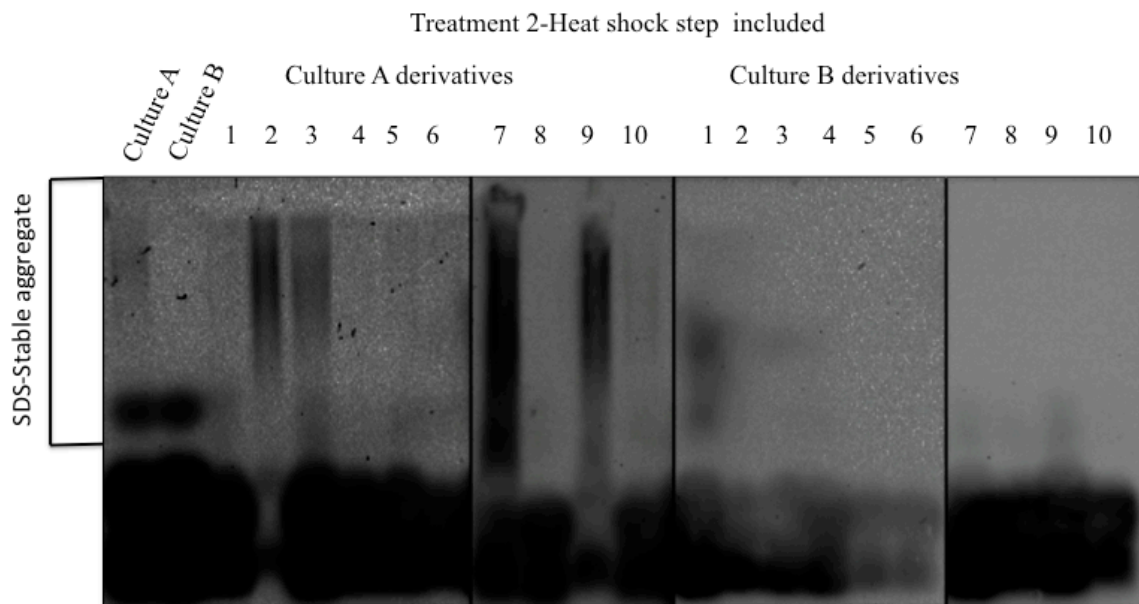


**Figure 2.3: Experimental design for studying prion heritability in *E. coli* under conditions that permit the removal of the *new1* gene.** NM aggregates are allowed to form in the bacterial cytoplasm in the presence of the New1-CFP fusion protein in Round 0. Heat shock then leads to removal of the *new1* gene from the bacterial chromosome. Cells are grown for 30 generations following *new1* removal and then analyzed for SDS-stable aggregates by SDD-AGE.

A.



B.



**Figure 2.4: Heat shock does not negatively affect the maintenance of NM aggregates in *E. coli*.** (A). SDD-AGE analysis of cultures from Round 1 of experimental design shown in Figure 2.1 (Cultures A and B were not subjected to a 15-minute heat shock step before being plated for single colonies). This gel image is a composite of separate gels. In the gel, the first two lanes contain cultures A and B, respectively. The next 10 lanes contain extracts from overnight cultures grown from 10

### Figure 2.4 continued

separate Round 1 colonies derived from the Culture A. Five out of ten samples (1, 3, 4, 7 and 9) contained NM aggregates. The next 10 lanes contain extracts from overnight cultures grown from 10 separate Round 1 colonies derived culture B. None of these samples contained detectable NM aggregates. The blot was probed with an anti-Sup35NM antibody. As previously mentioned, we can distinguish the signal present in the culture B derivatives, lanes 1 and 2, from *bona fide* SDS-stable aggregates, because this signal does not have distinct edges and does not follow the contours of the lanes.

(B) SDD-AGE analysis of cultures from Round 1 of experimental design shown in Figure 2.1, except that the starter cultures A and B were subjected to a 15-minute heat shock step before being plated for single colonies. This gel image is a composite of separate gels. In the gel, the first two lanes contain culture A and B. The next 10 lanes contain extracts from overnight cultures grown from 10 separate Round 1 colonies derived from the culture A. Nine out of ten samples (1-7 and 9, 10) contained NM aggregates although these aggregates are represented in weak smears in the extracts of lanes 1, 4, 5, 6 and 10. The next 10 lanes contain extracts from overnight cultures grown from 10 separate Round 1 colonies derived from culture B. None of these samples contained detectable NM aggregates. The blot was probed with an anti-Sup35NM antibody. For the reasons discussed before, the signal present in lane 1 of the culture B derivatives does not represent NM SDS-stable aggregates.

derived from culture A, nine contained NM aggregates (Nr.1-7 and 9, 10) (Fig. 2.4B).

None of the cultures derived from culture B contained detectable NM aggregates.

This experiment suggests that subjecting the cultures to heat shock does not negatively affect the heritability of the NM aggregates. In fact, heat shock correlates with a slight increase of the fraction of NM aggregates among the cultures inoculated with Round 1 colonies. Specifically, 9 out of 10 colonies contained NM aggregates in the case of heat shock treatment compared to 5 out of 10 colonies when we did not subject the cultures to heat shock (Fig. 2.4A, B).

To determine whether heat shock negatively affects the infectious character of the NM aggregates observed in culture A derivatives, we used the material from culture A derivative Nr.1 (Fig. 2.4B) to transform  $[pin^-][psi^-]$  yeast cells and compared the frequency of  $[PSI^+]$  transformants to the frequency of transformants obtained when using material from a sample that had not been subjected to heat shock following formation of NM aggregates (culture A derivative Nr.9 in Fig 2.4A). Extracts from culture A and from culture B derivative Nr.5 in Fig 2.4B served as positive and negative controls, respectively.

Our analysis showed that heat shock did not seem to negatively affect the infectivity of the NM aggregates. Specifically, the frequency of  $[PSI^+]$  yeast transformants obtained using heritable material generated in the absence of the heat shock step (0.5% using extract from culture A derivative Nr.9 in Fig 2.4A) was comparable to that obtained when using heritable material generated after the heat shock step (0.42% using extract from culture A derivative Nr.1 in Fig 2.4B) (Table 2.2). The extract from culture A resulted in



**Table 2.2: Heat shock does not negatively affect the infectivity of NM aggregates in *E.coli*.** Extracts from bacterial cultures shown in the table above were used to transform  $[pin^-][psi^-]$  yeast cells. The  $[psi^-]$  to  $[PSI^+]$  conversion percentages for the yeast transformants are shown. Analysis of these data by Fisher's exact test suggests that the observed difference in the frequencies of  $[PSI^+]$  transformants is statistically significant ( $P < 10^{-4}$ ).

Bacterial Extract	% of $[PSI^+]$ Transformants
Culture A	2.4% (147/6212)
Culture B derivative Nr.5-Fig 2.4B	0% (0/7748)
Culture A derivative Nr. 9-Fig 2.4A	0.5% (27/5480)
Culture A derivative Nr. 1 Fig 2.4B	0.42% (43/10320)

2.4% [*PSI*<sup>+</sup>] yeast transformants, while the extract from the negative control (culture B derivative Nr.5 in Fig 2.4B) did not result in any [*PSI*<sup>+</sup>] yeast transformants, which is consistent with the absence of NM aggregates in this sample.

**The infectious NM prion aggregates are maintained in the bacterial cytoplasm for up to 90 generations following PIN excision.**

Having now established that heat shock does not seem to hamper the heritability or infectivity of the NM aggregates, we investigated the maintenance of the NM aggregates in *E. coli* following excision of *newI* from the chromosome. Note that, in this experiment, the starter cultures in Round 0 also contained the plasmid carrying the temperature inducible enzymes needed for the removal of *newI* from the chromosome.

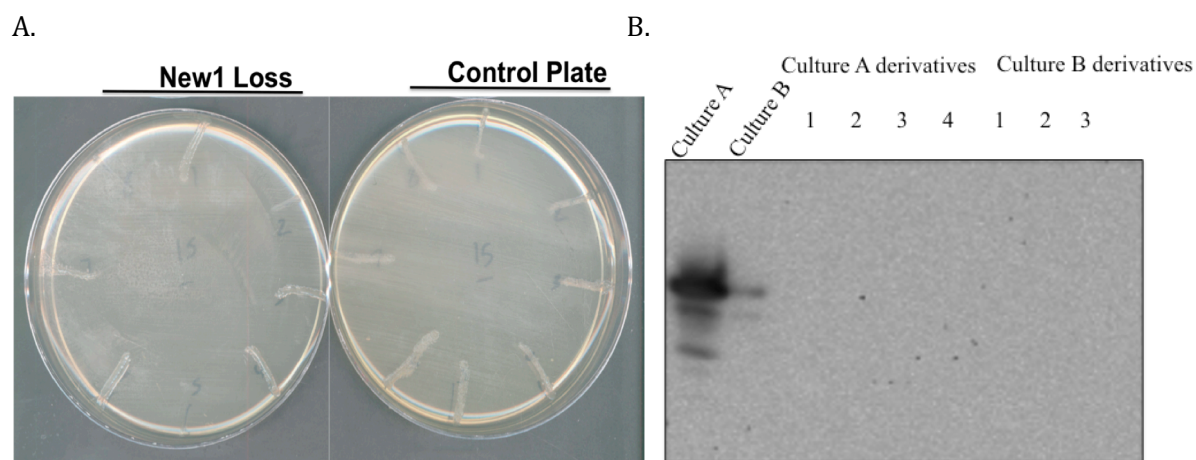
To carry out this experiment, we subjected the cells to a 15-minute heat shock to induce expression of the plasmid-encoded excision genes. This step allowed for the excision of the *newI* gene from the bacterial chromosome. We then plated the cells on medium containing arabinose, but not IPTG, to allow for continued synthesis of the NM-YFP fusion protein. Individual colonies were picked and inoculated into liquid medium for overnight growth (see Fig. 2.3 for experimental design). In parallel, the same individual colonies were tested for loss of the *newI* gene by patching them on kanamycin containing medium (see Fig. 2.5A). (Note that the cassette containing the *newI* gene also contains a linked kanamycin resistance marker.) In this experiment, the efficiency of *newI* gene loss was 50% (4 colonies out of 8 colonies tested), but the loss of *newI* was much less efficient in a subsequent experiment (see experiment described in Fig. 2.8). Cell extracts were then prepared from the overnight cultures grown from the individual kanamycin-sensitive colonies and analyzed by Western blot to confirm the absence of the

New1-CFP fusion protein (Fig. 2.5B). Our analysis showed that kanamycin sensitivity, corresponding to the loss of *newI* gene, correlated exactly with loss of any detectable New1 protein by Western blot.

To investigate whether the colonies that lost the *newI* gene contain NM aggregates, we prepared extracts from the overnight cultures of these individual colonies and analyzed them by means of SDD-AGE (Fig. 2.6A). We examined 4 samples derived from culture A (following excision of *newI*) and 3 samples derived from culture B. Of the 4 samples derived from culture A, two contained NM aggregates (Nr.1 and 4) (Fig 2.6A). None of the cultures derived from the culture B contained detectable NM aggregates. These same samples were also subjected to Western blot analysis in order to assess the NM-YFP fusion protein levels (Fig. 2.6B). We observed considerable variability in the levels of full-length NM-YFP protein between samples from either culture A or B derivatives as mentioned previously.

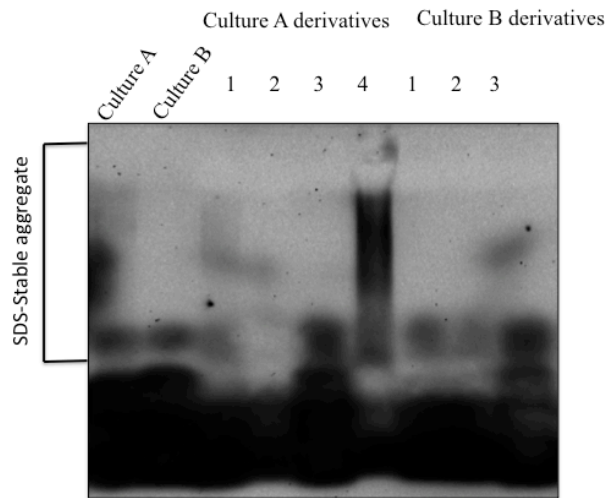
These results suggest that infectious NM aggregates can be maintained in the bacterial cytoplasm for up to 30 generations following *newI* excision. Additionally, these aggregates represent infectious material; when the aggregate-containing extract (specifically, culture A derivative Nr.4) was used to transform [*pin*<sup>-</sup>][*psi*<sup>-</sup>] yeast cells, 0.32% [*PSI*<sup>+</sup>] yeast transformants were obtained and this frequency was consistent with the frequency of [*PSI*<sup>+</sup>] yeast transformants observed when using extract from culture A (0.48% [*PSI*<sup>+</sup>] yeast transformants).

We next sought to determine whether the infectious NM aggregates could be maintained for a greater number of generations in *E. coli* following excision of the *newI*

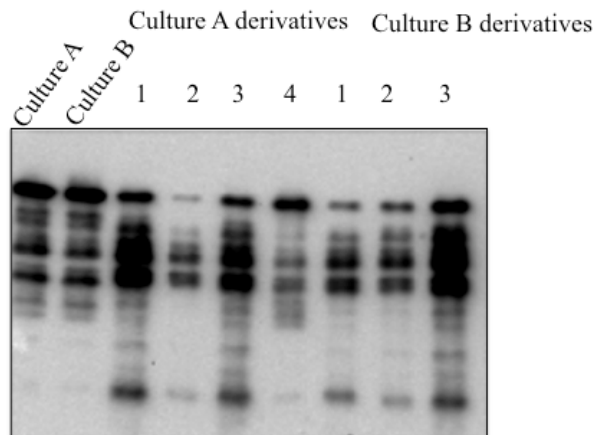


**Figure 2.5: Confirming *new1* removal from the chromosome.** (A) Confirming *new1* loss by plating on appropriate indicator medium. The *new1* gene in the *E. coli* chromosome is closely linked to a kanamycin resistance gene and the excision reaction results in the loss of both genes. Thus, kanamycin sensitivity (candidates 5 and 8) serves as a surrogate for loss of the *new1* gene. Candidates are numbered clockwise with candidate 1 at the 12 o'clock position. Both plates contain arabinose to induce the continuing synthesis of the NM-YFP fusion protein; in addition the test plate, but not the control plate, contains kanamycin. (B) Confirming the loss of New1 by Western analysis. The blot was probed with anti-HA antibody (The New1 protein is HA tagged). Lanes 1 and 2 contain cultures A and B, respectively. The next 4 lanes contain extracts from overnight cultures grown from 4 separate Round 1 colonies derived from culture A, each of which had lost the *new 1* gene based on kanamycin sensitivity. The last 3 lanes contain extracts from overnight cultures grown from 3 separate Round 1 colonies derived from culture B, each of which had similarly lost the *new1* gene.

A.



B.

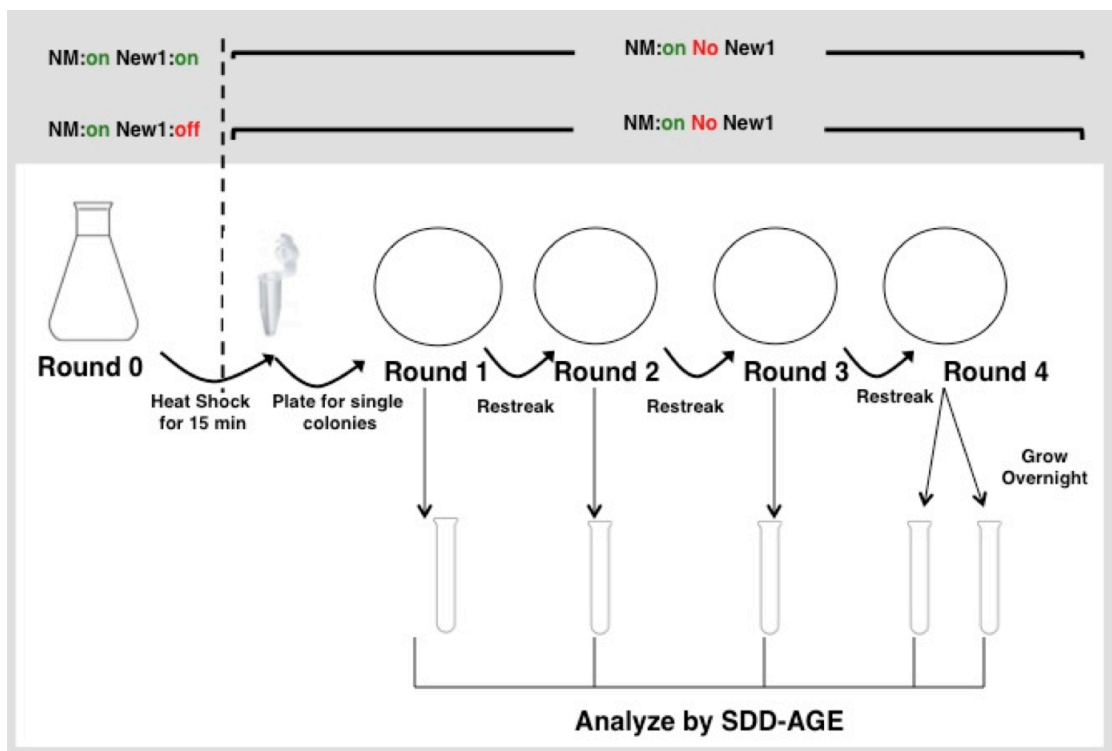


**Figure 2.6: Results of heritability experiments following *newI* removal from the chromosome.** (A) SDD-AGE analysis of cultures from round 1 of experimental design shown in Figure 2.3. Lanes 1 and 2 contain extracts from culture A and B respectively. The next 4 lanes contain extracts from overnight cultures grown from 4 separate Round 1 colonies derived from culture A, each of which had lost the *new I* gene based on kanamycin sensitivity. Two of these samples (1, 4,) still contained NM aggregates although *newI* has been removed from the chromosome. The last 3 lanes contain extracts from overnight cultures grown from 3 separate Round 1 colonies derived from culture B, each of which had lost the *newI* gene based on kanamycin sensitivity. None of these samples contained detectable NM aggregates. The blot was probed with an anti-Sup35NM antibody. (B) Western analysis of NM protein levels of the samples in (A). The blot was probed with an anti-Sup35NM antibody. The top band corresponds to the NM-YFP fusion protein. The bands observed below the full-length fusion protein represent degradation products.

gene. Our plan was to allow for additional rounds of cell division before analyzing cell extracts for presence of NM aggregates. To accomplish this, we restreaked individual colonies from Round 1 for single colonies before inoculating them into liquid medium for overnight growth. Each restreak increased the total generation number by about 20 generations (see Figure 2.7 for experimental design). At each round, we picked individual colonies and restreaked them further for single colonies and at the same time, inoculated them into liquid medium for overnight growth. Cell extracts were then prepared from these overnight cultures and analyzed for the presence of NM aggregates by SDD-AGE. This approach allowed us to investigate the presence of NM aggregates at about 20-generation intervals following excision of the [*PIN*<sup>+</sup>] factor.

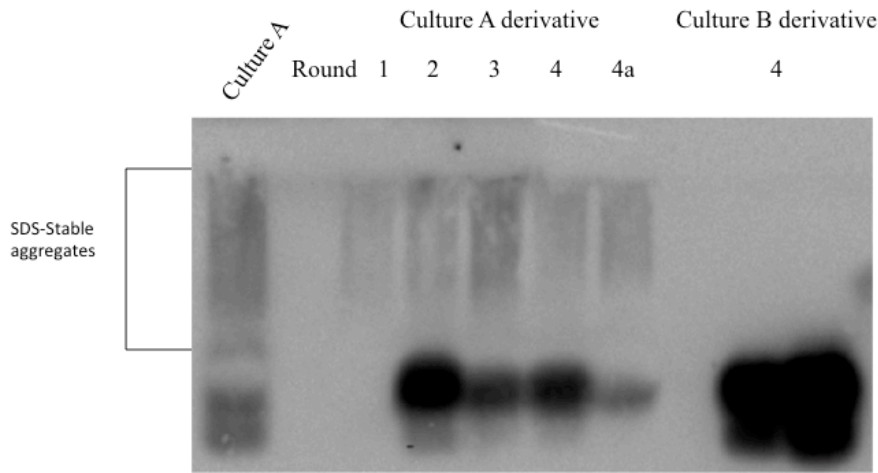
In this experiment, we analyzed 120 colonies in Round 1 for loss of the *newI* gene and obtained only five consisting of cells that had successfully excised the *newI* gene. We proceeded with these five Round 1 colonies, passaging them as depicted in Figure 2.7. We prepared bacterial extracts from overnight cultures grown from Round 1, 2, 3, and 4 colonies and analyzed them for presence of NM aggregates. Out of the five original Round 1 colonies, one maintained NM aggregates even after the third restreaking event (see Fig. 2.8 for SDD-AGE and Western analysis of the extracts prepared from the overnight cultures of this colony after each round). In parallel, we also analyzed five culture B derivatives that had been confirmed to have lost the *newI* gene.

For reasons we do not currently understand, four of the five Round 1 colonies derived from culture A that we examined gave rise to cultures that contained very low levels of NM protein (in some cases undetectable) at various stages of the restreaking process. This drop in NM protein level correlated with a loss of NM aggregates as assayed by

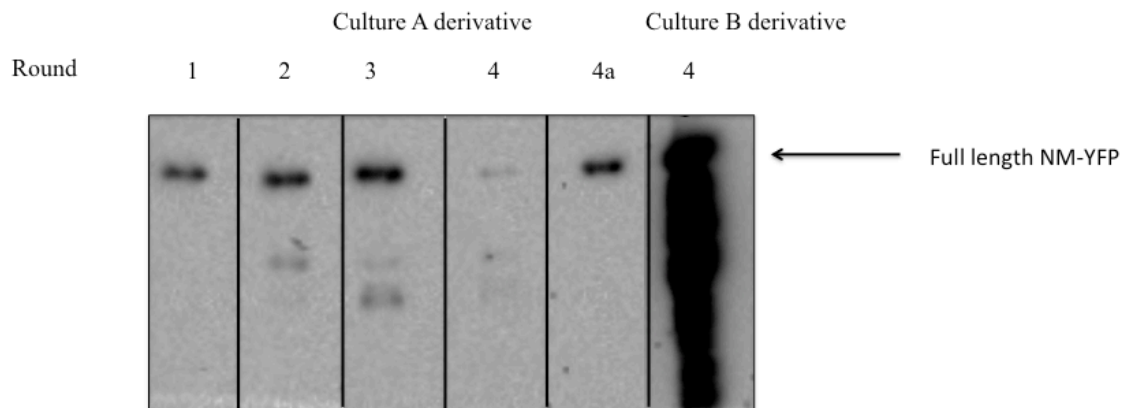


**Figure 2.7: Experimental design for studying prion heritability in *E. coli* following additional rounds of restreaking after *new1* removal.** NM aggregates are allowed to form in the bacterial cytoplasm in the presence of the New1-CFP fusion protein in Round 0. Heat shock then leads to removal of the *new1* gene from the bacterial chromosome. Cells are grown for up to 90 generations following *new1* removal and extracts are analyzed for SDS-stable aggregates at 20-generation intervals.

A.



B.



**Figure 2.8: Results of heritability experiments after 3 additional rounds of restreaking following *new1* removal.** (A) SDD-AGE analysis of extracts derived from each successive round of restreaking for the Round 1 clone that maintained NM aggregates. Lane 1 contains the starter culture A. Culture B is not shown in this gel but see Fig. 2.9B for analysis of this culture B. The next 4 lanes contain extracts from overnight cultures inoculated with individual colonies from Rounds 1-4 of the one sample that maintained the NM aggregates following all additional rounds of restreaking. The next lane, labeled 4a, contains extract from an overnight culture inoculated with another colony from the same Round 4 plate used to inoculate the for extract 4 culture. The last two lanes, labeled culture B derivative, contain a 1:10 and a 1:20 dilution of the extract from the overnight culture of a culture B derivative from Round 4. We ran dilutions of this extract because, as seen by the Western Blot analysis in (B), this sample had considerably more protein than the other samples loaded on the



### **Figure 2.8 continued**

same gel. This extract contained no detectable NM aggregates. The blot was probed with an anti-Sup35NM antibody. (B) Western analysis of extracts examined in (A). This gel image is a composite of separate gels. The first 4 lanes contain extracts from overnight cultures inoculated with individual colonies from Rounds 1-4 of the one sample that maintained the NM aggregates following all additional rounds of restreaking. The next lane, labeled 4a, contains extract from an overnight culture inoculated with another colony from the same Round 4 plate used to inoculate the extract 4 culture. The last lane, labeled culture B derivative, contains extract from the overnight culture of a culture B derivative from Round 4. As seen, this extract contained significantly more NM full-length protein than the rest of the samples.

SDD-AGE. For some of these clones, the amount of NM protein increased to high levels in the following rounds of restreaking, but NM aggregates were no longer observed (see SUPPLEMENT to CHAPTER 2 for SDD-AGE and Western blot analysis of extracts from the culture A and B derivatives from each stage of the experimental approach shown in Figure 2.7).

To investigate whether the NM aggregates from Rounds 1-4 shown in Figure 2.8A represent infectious material, we used the material to transform  $[pin^-][psi^-]$  yeast. Our analysis showed that, although the percentage of  $[PSI^+]$  yeast transformants varied from less than 1% to ~5% when using bacterial extracts obtained from different rounds, NM aggregates from each of the rounds represented infectious material (see Table 2.3). The extract from culture A resulted in 0.69%  $[PSI^+]$  yeast transformants, while the extract of the culture serving as a negative control, the extract of a culture B derivative from Round 4, did not result in any  $[PSI^+]$  yeast transformants. Interestingly, here we observed weak as well as strong  $[PSI^+]$  strains among the  $[PSI^+]$  yeast transformants [19].

These results indicate that once formed in the presence of New1 aggregates, NM aggregates can be maintained in the *E. coli* cytoplasm for about 90 generations following *new1* excision. Moreover our findings indicate that these NM aggregates correlate with the presence of infectious material that is capable of converting  $[pin^-][psi^-]$  yeast cells to  $[PSI^+]$ .

**Table 2.3: Infectivity of NM aggregates maintained in *E. coli* for 3 additional rounds of restreaking following *newI* removal from the chromosome.** Extracts from bacterial cultures shown in the table above were used to transform [*pin*]<sup>-</sup>[*psi*]<sup>-</sup> yeast cells. The [*psi*]<sup>-</sup> to [*PSI*]<sup>+</sup> conversion percentages for the yeast transformants are shown. The % [*PSI*]<sup>+</sup> weak and strong shown in the table are obtained by dividing the number of weak or strong [*PSI*]<sup>+</sup> transformants by the total number of transformants. The total % [*PSI*]<sup>+</sup> is obtained by adding the number of weak and strong [*PSI*]<sup>+</sup> transformants and dividing by the total number of transformants. Analysis of these data by Fisher's exact test suggests that the observed difference in the frequencies of [*PSI*]<sup>+</sup> transformants is statistically significant (P<10<sup>-2</sup>).

<b>Bacterial Extract</b>	<b>Total Nr.</b>	<b>Weak [PSI+] Nr.</b>	<b>Weak [PSI+] %</b>	<b>Strong [PSI+] Nr.</b>	<b>Strong [PSI+] %</b>	<b>Total %</b>
<b>Culture A</b>	3048	0	<b>0.00</b>	21	<b>0.69</b>	<b>0.69</b>
<b>Culture A derivative-Round 1</b>	9000	15	<b>0.17</b>	43	<b>0.48</b>	<b>0.64</b>
<b>Culture A derivative-Round 2</b>	3698	8	<b>0.22</b>	66	<b>1.78</b>	<b>2.00</b>
<b>Culture A derivative-Round 3</b>	3048	31	<b>1.02</b>	138	<b>4.53</b>	<b>5.54</b>
<b>Culture A derivative-Round 4</b>	2720	15	<b>0.55</b>	65	<b>2.39</b>	<b>2.94</b>
<b>Culture A derivative-Round 4a</b>	1800	21	<b>1.17</b>	50	<b>2.78</b>	<b>3.94</b>
<b>Culture B derivative-Round 4</b>	1200	0	<b>0</b>	0	<b>0</b>	<b>0</b>
<b>Plasmid Alone</b>	3232	0	<b>0</b>	0	<b>0</b>	<b>0</b>
<b>Buffer</b>	0	0	<b>0</b>	0	<b>0</b>	<b>0</b>

**Table 2.4 Plasmids and strains used for this study**

Strain/Plasmid	Relevant Characteristics	Source
<b>Plasmid</b>		
pSG241	<i>bla</i> P <sub>BAD</sub> <i>sup35NM<sup>WT</sup>-yfp</i> pBR322 ori; produces Sup35NM <sup>WT</sup> (residues 1-253) fused to YFP	[17]
pSG378	<i>URA3</i> pACYC184 ori <i>cat</i> <i>CEN6</i> ; shuttle vector pRS316 modified to contain pBR322-compatible origin and chloramphenicol resistance	[17]
pEN184	<i>cat</i> <i>lambdacI857 (ts)</i> <i>lambda pR-xis</i> <i>lambda in repA101 (ts)</i> ori <i>R101</i> ; pAH57 modified to contain chloramphenicol resistance	This study
<b>Strain (<i>Escherichia coli</i>)</b>		
SG811	BW27785 <i>attB::ahp lacIq</i> P <sub>tac</sub> <i>newI<sub>50-100</sub>-cfp-3xha</i> ; produces New1 (residues 50-100) fused to CFP and 3 HA tags from a chromosomal construct integrated at lambda attachment site	[17]
<b>Strain (<i>Saccharomyces cerevisiae</i>)</b>		
SG775	YJW187 [ <i>pin</i> <sup>-</sup> ]; derived by serial passage on YPD with 3mM GuHCl; phenotypically [ <i>pin</i> <sup>-</sup> ] [ <i>psi</i> <sup>-</sup> ];	[17]
SG861	YJW187 [PSI <sup>+</sup> ]; phenotypically weak [PSI <sup>+</sup> ]	[17]
SG862	SG775 [PSI <sup>+</sup> ]; phenotypically strong [PSI <sup>+</sup> ]	[17]

## DISCUSSION

The experiments presented in this chapter have demonstrated that the *E. coli* cellular environment supports the propagation of the prion form of the yeast Sup35 protein (its prion-forming domain, NM, in particular). NM aggregates formed in the presence of the PIN factor can be maintained in the bacterial cytoplasm for close to 90 generations following removal of PIN from the system, and these aggregates are infectious when used to transform yeast. It is interesting that the relation of PIN to NM aggregates in bacteria recapitulates the relationship in yeast with respect to both NM aggregate formation and aggregate propagation; namely, PIN is required for the *de novo* appearance of  $[PSI^+]$  but not for its propagation.

In yeast cells, once formed, the  $[PSI^+]$  prion is stably maintained. However, the stability of the NM prion aggregates formed in the bacterial cytoplasm remains to be investigated. At the present time, the fluctuation in the NM-YFP protein levels throughout the course of the experiment hampers our ability to investigate the stability of NM prion aggregates in the bacterial cytoplasm. Due to this problem, the system we use to study yeast prion heritability in the bacterial cytoplasm will need to be modified before the stability of NM aggregate propagation in *E. coli* can be assessed. To accomplish this, we suggest using a different induction system for NM gene expression, one that results in consistent NM protein levels throughout the experiment and is reproducible from experiment to experiment. In addition to answering the question of the stability of NM aggregates in *E. coli*, the new system would allow us to search for mutants that either increase or decrease the observed stability of the NM aggregates in *E. coli*.

Additionally, the system we employ to remove *newI* from the chromosome following formation of NM aggregates is not very efficient. In certain instances, the *newI* removal efficiency can be as low as 4% (see experiment described in Fig. 2.8), thus limiting the number of bacterial colonies we can analyze for heritable NM aggregates. To address this limitation, we suggest redesigning the system to provide *newI* on a plasmid with a temperature sensitive replicon that should allow for easy removal once the NM aggregates have formed.

Despite all the problems and limitations with the current system, we have shown that it is possible for infectious NM aggregates to be maintained for 90 generations in the bacterial environment. The general model developed to study prion heritability in the *E. coli* cytoplasm could also be used to test the effect specific bacterial chaperones have on this process. Preliminary work by a former member of our lab, Dr. Sean Garrity, has indeed shown that changes in the bacterial chaperone environment can alter the nature of the NM aggregates that are formed. Specifically, overproduction of ClpB, the Hsp104 bacterial homolog, not only seems to reduce the average size of the Sup35NM polymers, but it also seems to increase the infectivity of such aggregates (S. J. Garrity, unpublished data).

Based on these preliminary results, it is of great interest to examine how bacterial chaperones influence the heritability of yeast prions formed in the bacterial cytoplasm. In fact, in preliminary experiments we have found that heat shock, which upregulates the production of many chaperones, seems to increase the heritability of the Sup35NM aggregates in *E. coli* (see Fig. 2.4).

The results of the experiments described in this chapter set the stage for examining the interaction of bacterial chaperones with yeast prion proteins and more specifically for determining how bacterial chaperones influence prion propagation.

## **MATERIALS AND METHODS**

### **Plasmids, Strains and Cell growth**

Bacterial cultures were grown overnight and then diluted to OD<sub>600</sub> 0.02 in LB supplemented with the appropriate antibiotics (Carbenicillin 100 µg/ml; Chloramphenicol 25 µg/ml, Kanamycin 50 µg/ml), grown for 30 minutes at 30°C and induced with the appropriate inducers (L-Arabinose at 0.0002% w/v; IPTG at 1mM) for about 5 hours. For the experiment described in Figure 2.8 following addition of inducers the cultures were grown overnight and the next day the culture were diluted tenfold and grown for about 2.5 hours in the presence of the appropriate antibiotics and L-Arabinose at 0.0002% w/v. Following heat shock, the cultures were plated for single colonies on plates supplemented with carbenicillin (100 µg/ml) and L-Arabinose (0.0002% w/v) and grown at 37°C for efficient loss of the pEN184 plasmid. Additional restreaks as shown in Figure 2.7 were grown at 30°C.

The NM-YFP construct was produced from pBR322-derived plasmids under the control of the arabinose-inducible promoter pBAD. The New1-CFP-3xHA was integrated in the chromosome and was produced under the control of an IPTG inducible promoter.

### **Bacterial Extract Preparation**

50ml or 5ml cultures were grown to the indicated times and the OD<sub>600</sub> was recorded. Bacterial extracts were then prepared as described [17] with the following modifications. Cell debris was removed from the lysate by centrifugation for 10 minutes at 500 x g at 4°C. Lysates were normalized for total protein as assayed by bicinchoninic acid (ThermoFisher).



### **Semi-Denaturing Detergent Agarose Gel Electrophoresis**

SDD-AGE was performed as described [21] using 1.5% agarose. Blots were probed with anti-HA (clone 3F10; Roche), anti-Sup35 yS-20 (Santa Cruz Biotechnology). Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rat IgG (Abcam ab6734), and protein G (Abcam ab7460). Blots were detected using ECLplus Western Blotting Detection System (GE Healthcare).

### **Protein Extract Transformations**

25  $\mu$ l of bacterial or yeast extract was used to transform 100  $\mu$ l of [*pin*<sup>-</sup>][*psi*<sup>-</sup>] yeast spheroplasts as described [17] (and reference therein [22]). [*PSI*<sup>+</sup>] transformants were scored as described [22].

### **Bacterial and Yeast Fusions**

Fusion of bacterial protoplast to [*pin*<sup>-</sup>][*psi*<sup>-</sup>] yeast spheroplasts was performed as described elsewhere [17] (and references therein [22, 23])

## **ACKNOWLEDGEMENTS**

We thank V. Sivanathan for valuable discussion and helpful advice. This work was supported by a National Institutes of Health Pioneer Award OD003806 (to A.H.).

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## **SUPPLEMENT TO CHAPTER 2**

### **Study of Sup35NM-YFP fusion protein levels**

## **ATTRIBUTIONS**

I performed the experiments presented in this supplement.

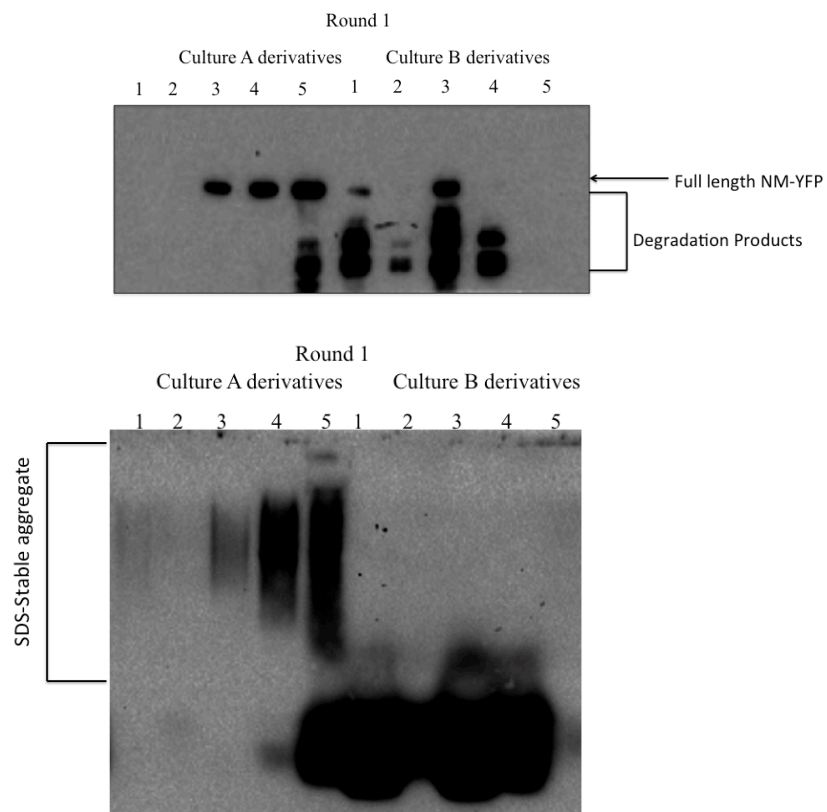
## RESULTS AND DISCUSSION

**The arabinose inducible system results in fluctuating levels of full-length NM protein from colony to colony as well as in the same clone over successive rounds of restreaking.**

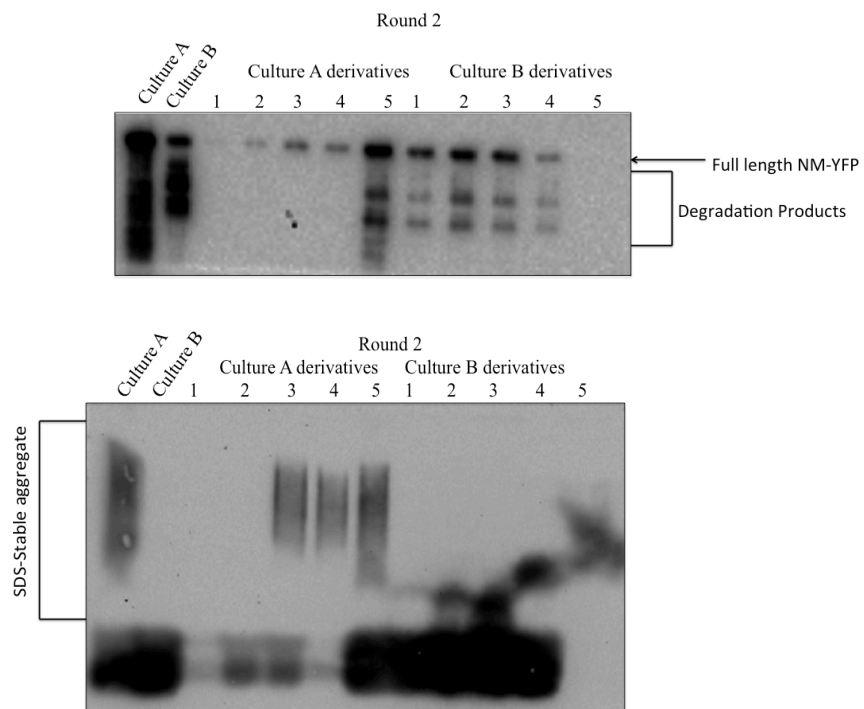
In the work presented in this Supplement, we follow the full-length protein levels and aggregative state of the NM-YFP fusion protein through the 3 rounds of restreaking shown in Fig.2.7. The samples shown in Fig. 2.8 of this CHAPTER were a subset of those discussed here. As described above, after the growth of starter cultures A and B (and the formation of NM aggregates in culture A), we subjected the cells to a 15-minute heat shock to induce the plasmid-encoded enzymes needed for the removal of *newI* from the bacterial chromosome. We then plated the cells on medium containing arabinose for the continued expression of the NM-YFP fusion protein and picked individual colonies to test for the loss of *newI*. We obtained 5 derivatives from each of the starter cultures that were confirmed to have lost the *newI* gene. We then carried these derivative colonies through the remaining rounds of the experimental design shown in Fig. 2.7 and analyzed cell extracts prepared after each round of restreaking by means of SDD-AGE and Western analysis for NM aggregates and protein levels, respectively.

Our analysis showed that in Round 1, only three (Nr.3, 4 and 5) of the 5 culture A derivatives contained detectable full-length NM protein and all three of these cultures contained NM aggregates as detected with SDD-AGE (Fig. 2.9A). Although culture A derivative Nr.1 contained NM aggregates and therefore must contain full-length NM protein, we did not detect the protein in our Western blot experiment.

A.



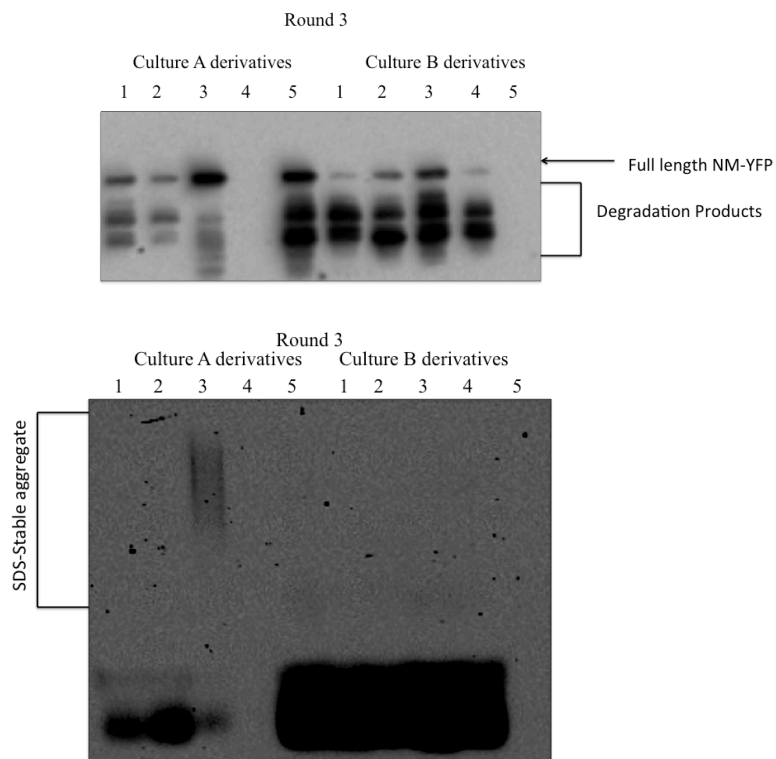
B.



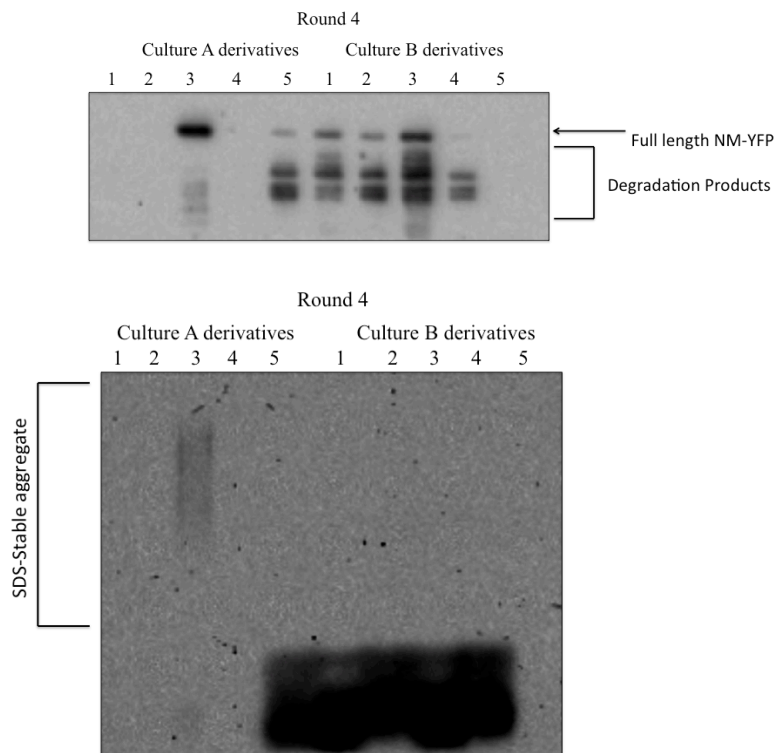
**Figure 2.9 Arabinose induction results in varying NM-YFP full-length protein levels from colony to colony and in the same clone over time.**



C.



D.



**Figure 2.9 continued**

### Figure 2.9 continued

(A) Western blot (top panel) and SDD-AGE analysis (bottom panel) of cultures from Round 1 of experimental design shown in Figure 2.7. The first 5 lanes contain extracts from overnight cultures grown from 5 separate Round 1 colonies derived from culture A. The last 5 lanes contain extracts from overnight cultures grown from 5 separate Round 1 colonies derived from culture B. None of the culture B derivatives contained detectable NM aggregates. The blots were probed with an anti-Sup35NM antibody. (B) Western blot (top panel) and SDD-AGE analysis (bottom panel) of cultures from Round 2 of experimental design shown in Figure 2.7. Lanes 1 and 2 contain extracts from cultures A and B, respectively. The next 5 lanes contain extracts from overnight cultures grown from 5 separate Round 2 colonies derived from culture A. The last 5 lanes contain extracts from overnight cultures grown from 5 separate Round 2 colonies derived culture B. None of the culture B derivatives contained detectable NM aggregates. The blots were probed with an anti-Sup35NM antibody. (C) Western blot (top panel) and SDD-AGE analysis (bottom panel) of the same cultures as in (A) and (B), but from Round 3 of experimental design shown in Figure 2.7. (D) Western (top panel) and SDD-AGE analysis (bottom panel) of the same cultures as in (A), (B) and (C), but from Round 4 of experimental design shown in Figure 2.7.

Only two (Nr.1 and 3) out of the 5 culture B derivatives contained detectable full-length NM protein, but neither of them contained any detectable aggregates.

In analyzing extracts from Round 2 (Fig. 2.9B) we observed that the pattern of full-length NM expression of the bacterial clones differed from the one seen in Round 1. For instance, culture A derivatives Nr.3, 4 and 5 still contained high levels of NM protein and also NM aggregates, but culture Nr.2 also had some detectable full-length NM protein, in contrast to what we saw in Round 1. However, this culture did not contain any NM aggregates in Round 2, possibly due to the fact that protein synthesis was interrupted in Round 1. Moreover, the PIN factor had been removed from the system and therefore no *de novo* NM aggregation could occur. Culture A derivative Nr.1 had also lost the NM aggregates in Round 2, possibly due to the fact that the very low levels of NM protein were not sufficient to support aggregation. Culture B derivatives Nr.2 and 4 now had detectable full length NM in addition to the cultures Nr.1 and 3. However, as expected, none of these cultures contained any detectable NM aggregates.

In Round 3 (Figure 2.9C), culture A derivatives Nr.1 and 2 contained detectable levels of NM protein, but as expected this upregulation in NM protein levels did not correlate with reappearance of NM aggregation due to the absence of PIN factor. Culture A derivatives Nr.3 and 5 still maintained high NM levels and the associated aggregates (though the signal was faint for culture Nr.5). However, culture A derivative Nr.4 no longer contained any detectable full-length NM protein, and it had also lost the NM aggregates. The protein levels for the culture B derivatives also had a slightly different pattern in this Round than the pattern seen in Round 2.

Finally, in Round 4 (Figure 2.9D), only culture A derivative, Nr.3, contained high levels of NM protein and the associated NM aggregates. Culture Nr.5 contained low levels of full-length NM and it no longer contained detectable NM aggregates, possibly due to the decrease in NM-YFP protein levels.

This experiment clearly shows that the current, arabinose inducible system is not optimal for the study of prion heritability in the bacterial cytoplasm. The observation that NM protein levels in the same clone can fluctuate significantly from round to round, sometimes even dropping below levels necessary to maintain an aggregated state, makes it impossible to draw conclusions about the stability of NM aggregate propagation in the *E. coli* system. We believe that the observed number of clones able to maintain NM aggregates through all four rounds of our experimental design is probably an underestimate, as some colonies lost the aggregates in the course of the experiment due to the interrupted synthesis of the NM-YFP fusion protein.

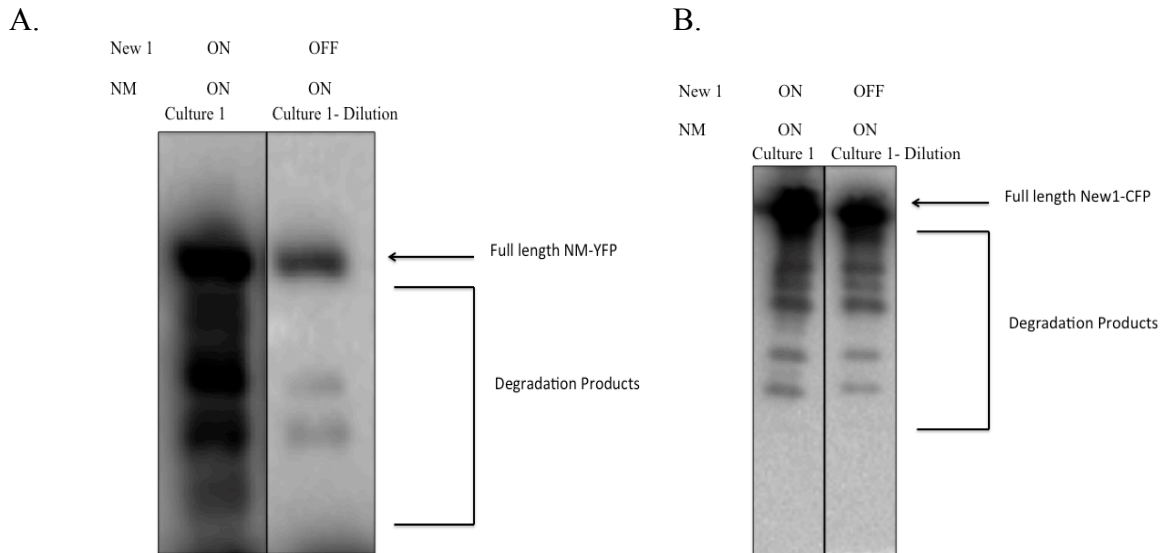
We obtained additional information about the conditions that lead to fluctuations in NM protein levels in the course of another experiment that was performed, in part, to investigate the effect of culture dilution on the NM-YFP protein levels. Previous evidence from the lab had suggested that dilution into fresh medium appeared to downregulate the synthesis of the NM-YFP fusion protein (S. J. Garrity, pers. comm.), an observation we wished to follow up on.

For this experiment, we grew bacterial cultures under inducing conditions for both NM and New1 for about 6 hours to an OD of 2 and then diluted the culture ten-fold into fresh medium supplemented with arabinose only for the continued induction of the NM fusion protein. As a result, the concentration of arabinose in the diluted culture remained

identical to that of the initial culture, while the concentration of the New1 inducer, IPTG, was reduced ten-fold. (We anticipated that this 10-fold reduction in IPTG concentration, from 1 mM to 100  $\mu$ M, would still leave enough IPTG in the culture to fully induce expression of the New1 fusion protein.) We analyzed extracts from both the initial and the diluted cultures for NM-YFP and New-CFP protein levels by means of a Western blot.

The Western blot revealed that the New1-CFP protein levels remained unchanged in the two cultures despite the IPTG concentration in the diluted culture being 1/10 of the concentration of the initial culture (see Figure 2.10B). However, we noticed a significant drop in the levels of the NM-YFP fusion protein in the diluted culture as compared to the initial culture even though the arabinose concentration did not change (see Figure 2.10A).

This experiment suggests that dilution into fresh medium results in a specific downregulation of NM-YFP levels. This observation suggests that, when passaged into fresh medium in the course of our experiment, the NM protein levels can initially drop to levels that may be insufficient for aggregate maintenance. Even if the NM protein levels are subsequently upregulated in the same culture, the NM aggregates would be irreversibly lost. In our experimental set-up, we routinely analyze liquid cultures for NM protein levels and NM aggregates at the end of their overnight growth, and although we sometimes notice high NM protein levels at this stage, we do not know the history of these protein levels during the course of the growth of the culture. Therefore, lack of aggregates even in the presence of high NM protein levels, could be due to the interruption of NM-YFP protein synthesis at some point during growth.



**Figure 2.10 Dilution into fresh medium leads to a drastic reduction in NM-YFP protein levels but does not affect the New1-CFP protein levels.** (A) Western analysis of NM-YFP levels in a dilution culture as compared to initial culture. NM-YFP levels dropped significantly when the culture was diluted into fresh medium although the inducer concentration remained unchanged. The blots were probed with an anti-Sup35NM antibody. (B) Western analysis of New1-CFP levels in a dilution culture as compared to initial culture. New1-CFP levels remained unchanged in the dilution culture although the inducer concentration dropped ten-fold when diluted. The blots were probed with an anti-HA antibody.

For the reasons discussed here, we believe that our system needs to be redesigned in order to study the stability of yeast prions in bacteria. We believe that using a different induction system for the NM-YFP fusion protein would eliminate the problem of fluctuating protein levels. Our analysis indicates that an IPTG inducible system results in a more reproducible and consistent protein synthesis (Fig. 2.10) and as a result might be better suited for the production of the NM-YFP fusion protein in the experimental set-up used to study yeast prion heritability in *E. coli*.

## **CHAPTER 3**

**Identifying cellular factors that influence amyloid formation in *E. coli***



## **ATTRIBUTIONS**

I performed the experiments presented in this chapter. Critical advice, reagents and directions on methods were provided by Dr. Sean J. Garrity. Helpful comments were provided by Viknesh Sivanathan.

## ABSTRACT

By showing that the bacterial cytoplasm can support the formation and maintenance of infectious prion aggregates, studies from this lab have shown that the bacterial cells can serve as a heterologous system in which to study prion biology. In yeast, cellular chaperones have been implicated in the *de novo* formation of prion aggregates, but the effect of the bacterial chaperone system on the formation of prion amyloids remains to be investigated. Using the non-prion, amyloid forming bacterial protein CsgB as a model protein, we have identified three *E. coli* open reading frames that, when overexpressed, potentially inhibit the formation of amyloid aggregates in the bacterial cytoplasm. The work presented here further supports the use of *E. coli* as a model system in studying the behavior of known and putative prion proteins.

## INTRODUCTION

Prions are infectious, self-propagating protein aggregates with a characteristic amyloid structure made up of beta-sheet-rich fibrils, where the beta-strands run perpendicular to the fibril axis [1, 2]. Prions were first described in mammals, where they cause a group of devastating neurodegenerative disorders called the transmissible spongiform encephalopathies. Subsequent work uncovered a prion-like phenomenon in yeast and other fungi. However, unlike mammalian prions, yeast prions do not result in cell death, but rather have been shown to act as protein-based hereditary elements that are stably maintained from generation to generation.

The discovery of prion forming proteins in yeast has dramatically improved our understanding of prion biology. Studies in yeast have shown that propagation of prion aggregates is critically dependent on the cellular chaperone machinery and specifically on the hexameric AAA<sup>+</sup> ATPase Hsp104 [3, 4]. This chaperone is thought to mediate the propagation of prion aggregates from generation to generation by breaking large aggregates into smaller units than can easily be passed on to daughter cells [5]. Deletion of Hsp104 results in a loss of the prion aggregates and their related phenotypes in yeast. In the case of Sup35, the best-characterized prion forming protein in yeast [6], overproduction of Hsp104 also results in a loss of the prion-associated phenotype, presumably due to the complete disaggregation of prion aggregates by Hsp104 [7, 8]. However, curing of the prion state by overproduction of Hsp104 has been observed only in the case of the Sup35 prion, but not in the case of other well-studied yeast prions [6].

Studies in yeast have also revealed a role for other cellular chaperones, including Hsp 40 and Hsp 70, not only in the process of prion propagation, but also in the *de novo*

appearance of prions [9-15]. However, the precise mechanisms by which these chaperones influence prion behavior in yeast have been very difficult to elucidate partly due to the fact that the same chaperone can have opposing effects on different prions and sometimes even on the same prion depending on the presence or absence of additional yeast chaperones [16, 17]. Dissection of the interactions of the yeast chaperone systems with yeast prions is also complicated by the fact that yeast cells encode several different proteins for each class of chaperones.

Although it is not yet known if bacteria contain prions, bacteria provide a potentially useful experimental system in which to study the behavior of known and putative prion proteins. Indeed, work from this lab (reference [18] and work presented in CHAPTER 2) has shown that the bacterial cytoplasm can support formation and even maintenance of yeast prions. Moreover, bacteria encode the homologs of chaperones shown to have an effect on prion behavior in yeast, and conveniently contain a reduced number of redundant chaperones and regulators compared to yeast. In addition, non-prion, amyloid-forming proteins have already been described in bacteria [19], though all known examples aggregate extracellularly, where they contribute to physiological functions such as biofilm formation, host colonization, cell invasion, and immune activation.

Under appropriate conditions, *E. coli* and *Salmonella* species elaborate cell surface-attached amyloid fibers known as curli that are an important structural constituent of biofilms. Unlike the production of disease-associated amyloids, curli fiber formation is not the result of protein misfolding but instead depends on a dedicated and highly controlled pathway. In *E. coli*, there are at least 7 proteins involved in curli formation and they are encoded by the two divergently transcribed operons *csgBAC* and *csgDEFG*.

CsgD is the master regulator of curli biogenesis and it is essential for the transcription of the *csgBAC* operon [20], which encodes the major and minor components of the fiber, CsgA and CsgB, respectively [21]. CsgG is secreted into the periplasm via its Sec signal sequence and from there it is transported into the outer membrane, where it polymerizes to form the pore required for the export of CsgA and CsgB into the extracellular space [22]. Like the other curli proteins, CsgA and CsgB are secreted into the periplasm via the Sec translocon. From there, with the help of the three additional curli proteins CsgC, CsgE and CsgF, they are directed through the CsgG pore to the extracellular space [20, 22, 23]. A C-terminal sequence in CsgB mediates its insertion into the outer membrane, and from that position, CsgB templates the amyloid polymerization of the freely secreted CsgA [24].

CsgB can also form cytoplasmic amyloid if its N-terminal signal sequence (Sec signal sequence) is removed [19, 25]. Studies by Dr. Sean Garrity, a former graduate student in our lab, demonstrated that when fused to the CI protein of bacteriophage lambda ( $\lambda$ CI; the lambda repressor) and produced in the cytoplasm, CsgB causes cell toxicity, which correlates with the ability of the CsgB moiety to form amyloid-like aggregates (a mutation in the CsgB moiety that impedes amyloid conversion relieves this toxicity) (S.J. Garrity, unpublished data). A sequence-specific DNA-binding protein that binds cooperatively to adjacent and non-adjacent operators on the phage chromosome,  $\lambda$ CI can also bind to lower affinity operator-like sequences on the bacterial chromosome when overproduced. Subsequent work by a postdoc in the lab, Dr. Viknesh Sivanathan, indicated that the toxicity of the  $\lambda$ CI-CsgB fusion protein was also relieved by mutations in the  $\lambda$ CI moiety that abolish cooperative DNA binding (V. Sivanathan, unpublished

data), suggesting that the toxicity of the fusion protein is due to both its amyloidogenicity and its non-specific DNA binding activity.

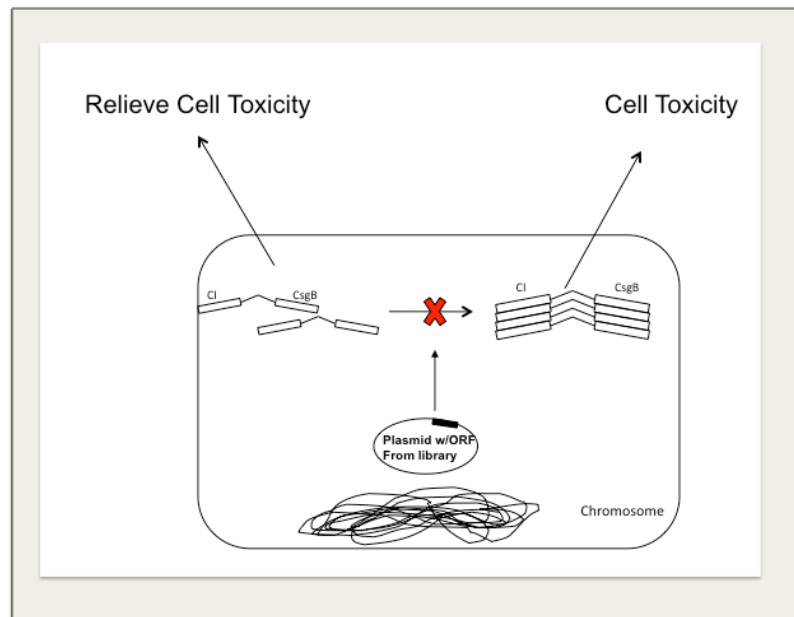
Here, we take advantage of the toxicity of the  $\lambda$ CI-CsgB fusion protein to design a selection system that allows us to identify open reading frames that influence amyloid formation in *E. coli*. Using relief of toxicity as a readout of decreased aggregating ability, we identified three open reading frames in *E. coli* that, when overexpressed, potentially affect cytoplasmic amyloid formation. We outline further experiments that could be performed to better characterize the effects of the proteins encoded by these open reading frames.

## RESULTS

### **Selection system used to identify bacterial factors that influence amyloid formation in *E. coli* and identification of candidate ORFs.**

Since work from this lab had already shown that the bacterial cytoplasm could support the formation and propagation of prion aggregates, we were interested in studying the effect of the bacterial chaperone system on the formation of prion amyloids. In fact, studies in yeast have implicated cellular chaperones in the *de novo* formation of the Sup35 prion, known as  $[PSI^+]$  [12, 13]. In our study, we used the amyloid aggregation of the curli component CsgB as a surrogate for the initial formation of prion-like aggregates.

As mentioned above, we took advantage of the cell toxicity of a  $\lambda$ CI-CsgB fusion protein to design a selection system to identify cellular factors that affect amyloid formation in *E. coli*. Using relief of toxicity as readout of decreased aggregating ability, we tested the effect of overexpressing a library of *E. coli* ORFs on the ability of the  $\lambda$ CI-CsgB fusion protein to access the amyloid conformation (see Figure 3.1 for experimental approach). To carry out this selection, we transformed a plasmid encoding the  $\lambda$ CI-CsgB fusion protein under the control of an IPTG-inducible promoter into the *E. coli* ASKA library, where each cell harbors a mobile plasmid carrying one of the 4300 *E. coli* ORFs also under the control of an IPTG-inducible promoter [26, 27]. We plated the transformants onto medium supplemented with the appropriate antibiotic and 125 $\mu$ M IPTG to induce the synthesis of both the  $\lambda$ CI-CsgB fusion protein and the protein encoded on the mobile plasmid. Using control strains from the ASKA library, we had already shown that the plating efficiency of cells transformed with the  $\lambda$ CI-CsgB plasmid

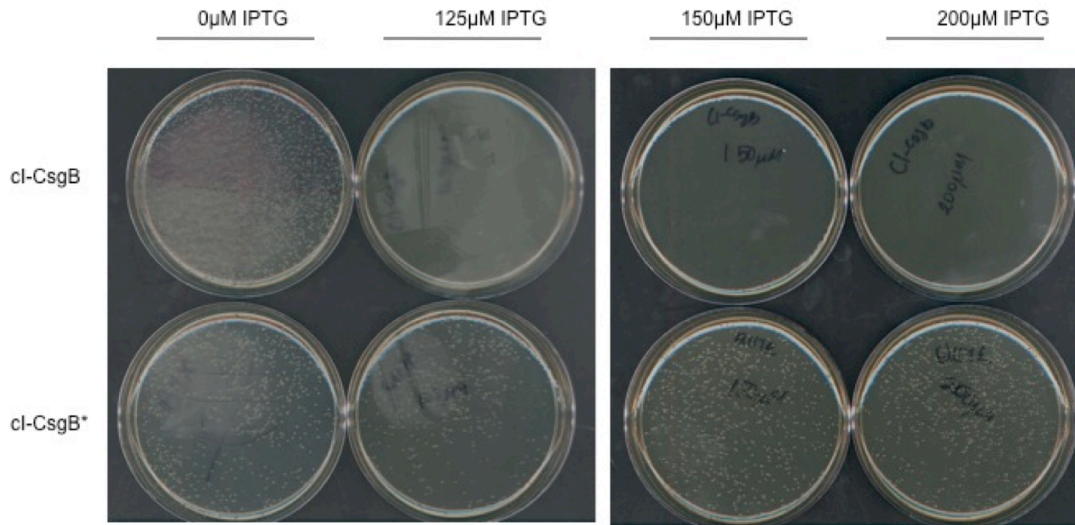


**Figure 3.1: Experimental design for identifying ORFs that affect amyloid formation *E. coli*.** Aggregation of the  $\lambda$ CI-CsgB fusion protein results in cell toxicity. Overexpression of an ORF inhibiting amyloid formation will result in relief of the amyloid mediated toxicity.



was 1/1000 to 1/3000 in the presence of 125 $\mu$ M IPTG, whereas the plating efficiency of cells transformed with an aggregation deficient version of the  $\lambda$ CI-CsgB fusion protein ( $\lambda$ CI-CsgB Q117R; S.J. Garrity, unpublished data) was  $\sim$ 1 (see Figure 3.2).

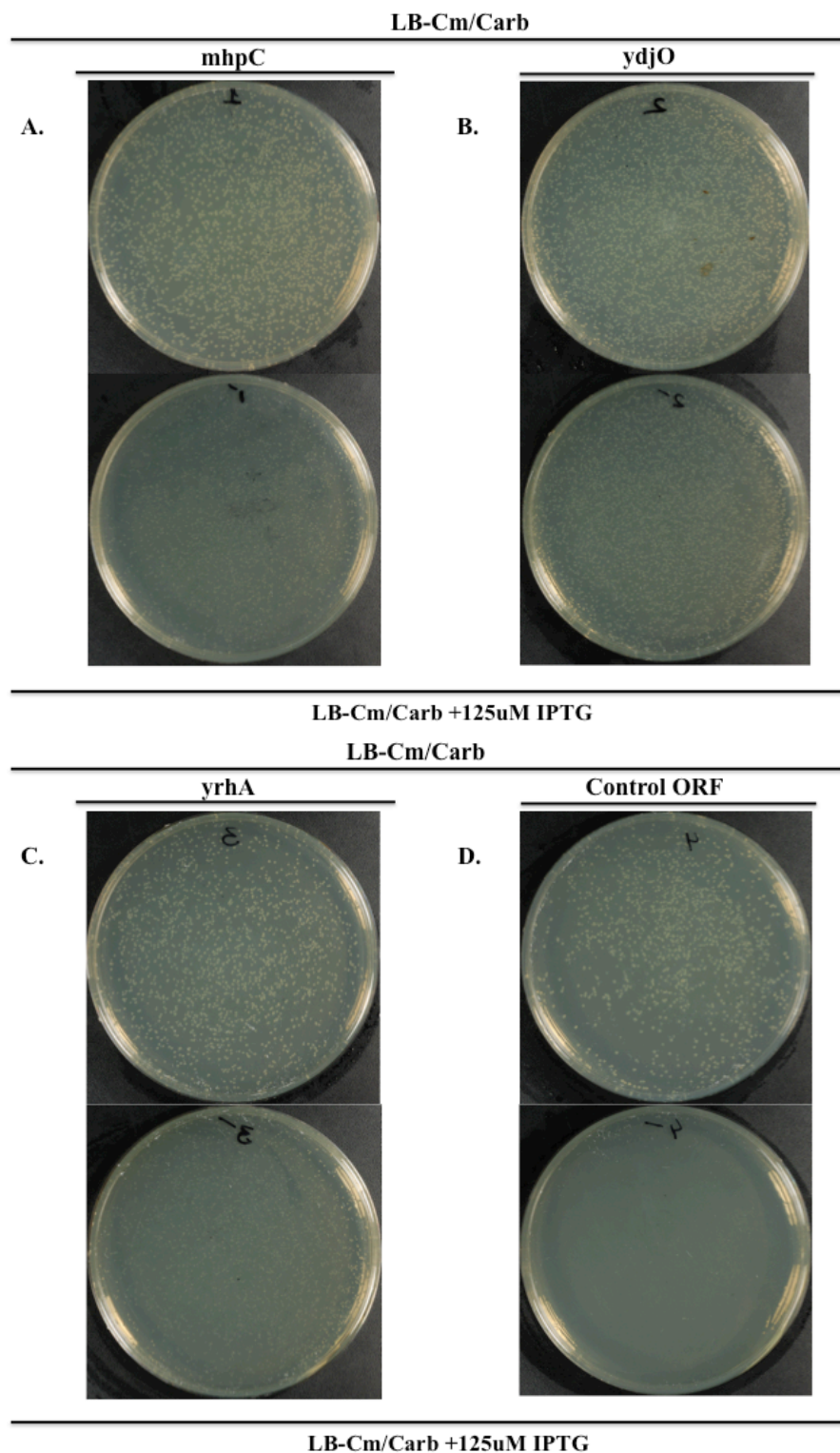
We identified 40 surviving colonies out of 13,000 transformants plated and restreaked them on IPTG containing plates to confirm the relief of toxicity. Out of the 40 surviving colonies, only 13 were confirmed by the restreak. Then, using universal primers that annealed to the backbone of the plasmid carrying the individual ORFs, we amplified and sequenced the ORF being expressed in each of the surviving colonies. Using this method we identified 6 different ORFs present in the surviving colonies. To confirm that the ORFs being expressed in these clones did indeed relieve the toxicity of the fusion protein, we re-transformed the  $\lambda$ CI-CsgB plasmid into the ASKA strain carrying the specific ORF identified in the initial selection. We were able to do this only for 5 out of the 6 ORFs identified, as we did not have access to the ASKA strain carrying one of the identified ORFs. Only 3 of the 5 identified *E. coli* ORFs relieved the toxicity of the fusion protein when re-tested in this manner (see Table 3.1 and Figure 3.3).



**Figure 3.2:  $\lambda$  CI-CsgB causes cell toxicity starting at an IPTG concentration of 125μM.** The JA200 strain overexpressing the *ycfD* open reading frame, transformed with either the  $\lambda$ CI-CsgB or the  $\lambda$ CI-CsgB Q117R plasmid, and grown on plates with the indicated IPTG concentration. As seen, the  $\lambda$ CI-CsgB plasmid causes cell toxicity at an induction level of 125μM IPTG, whereas the  $\lambda$ CI-CsgB Q117R plasmid does not.

**Table 3.1: A list of the clones obtained in the selection using relief of toxicity as a readout of decreased aggregating ability.** The table indicates the name of the selected clone, the name of the ORF overexpressed in that clone and a description of the ORF.

Clone Name	ORF Name	Function of ORF
<b>MOB-8</b>	mhpC	2-hydroxy-6-ketono-2,4-dienedioate hydrolase
<b>MOB-12</b>	ydjO	Predicted protein
<b>MOB2-27</b>	yrhA	Conserved Protein



**Figure 3.3: Reconfirmation of toxicity relief by ORFs identified in the selection.** The ASKA clones overexpressing the ORF identified in the initial selection were re-transformed with the  $\lambda$ CI-CsgB plasmid and grown on medium lacking or containing

**Figure 3.3 continued**

IPTG. As seen, the ORFs identified in the selection relieve  $\lambda$ CI-CsgB-induced toxicity. Shown are the results for (A) *mhpC*, (B) *ydjO*, (C) *yrhA* and (D) a control ORF, *yciE*.

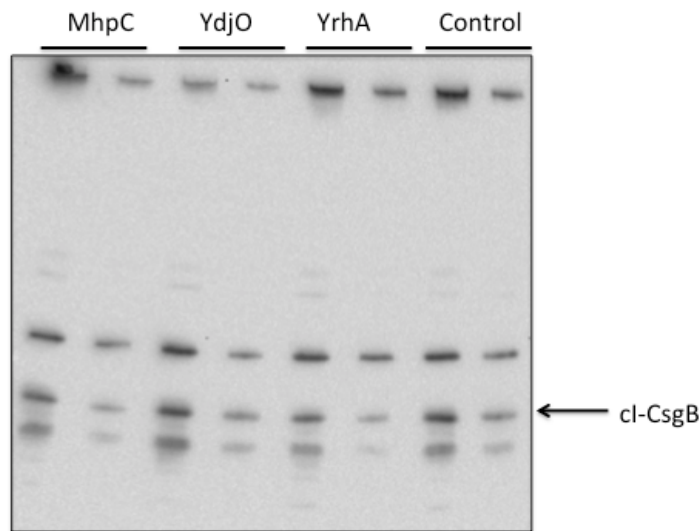
## Initial validation of candidate ORFs

One possible way for the candidate ORFs identified in our selection to relieve  $\lambda$ CI-CsgB-mediated toxicity would be to cause a drop in fusion protein levels (either by downregulating fusion protein synthesis or by destabilizing the fusion protein). In this scenario, the relief of toxicity would merely be due to the fact that the intracellular concentration of fusion protein is too low to permit sufficient amounts of amyloid material to form. To determine whether the ORFs identified in our selection relieve toxicity by altering fusion protein levels, we compared the  $\lambda$ CI-CsgB levels in cells overexpressing the identified ORFs to those in cells overexpressing a control ORF that did not relieve fusion protein toxicity. It was also essential that the control ORF chosen did not cause cell toxicity on its own, in the absence of  $\lambda$ CI-CsgB.

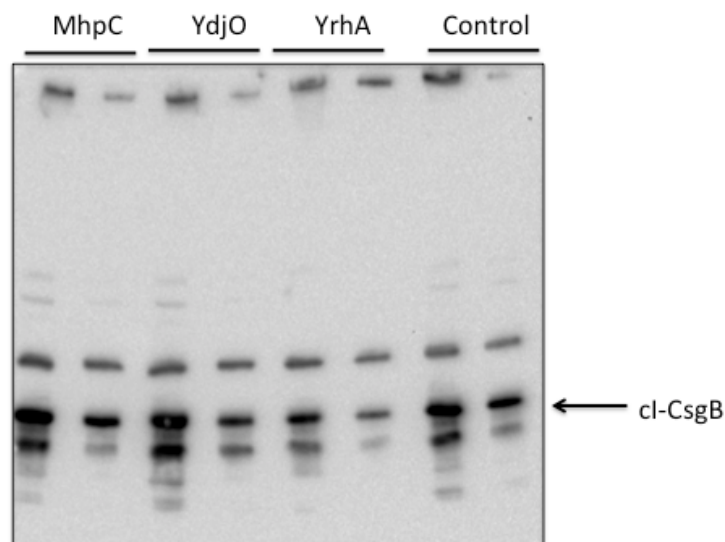
We could not carry out this experiment simply by culturing the cells in the presence of IPTG (to maintain synthesis of both the  $\lambda$ CI-CsgB fusion protein and the ORF-encoded protein) because, whereas bacterial cultures containing one of the selected ORFs would grow normally under these conditions, the cultures overexpressing the control ORF would not since the aggregation of the  $\lambda$ CI-CsgB fusion protein would cause cell toxicity. To solve this problem, we cultured the cells (co-transformed with both the ORF plasmid and the  $\lambda$ CI-CsgB plasmid) under non-inducing condition until they reached an optical density (OD) of about 0.8. We then added inducer to the cultures and let them grow for an additional 30 minutes or 1 hour. This time-frame allowed the cells to produce sufficient  $\lambda$ CI-CsgB to be detectable on a Western blot before significant toxicity was observed. The results of the Western analysis, performed with samples harvested at both the 30-minute and the 1-hour time point, indicated that the  $\lambda$ CI-CsgB fusion protein

levels in cell cultures overexpressing the experimental ORFs are similar to the levels seen in cell cultures overexpressing a control ORF (see Figure 3.4). These findings identify the MhpC, YdjO and YrhA proteins as worthwhile candidates to further characterize for their possible effects on CsgB amyloid formation in the *E. coli* cytoplasm.

A.



B.



**Figure 3.4. The ORFs identified in the selection do not downregulate  $\lambda$ CI -CsgB protein levels.** (A) Western analysis of bacterial cultures expressing the indicated ORF and  $\lambda$ CI-CsgB, grown for about 3 hours, induced with IPTG and grown for an additional 30 minutes. The two lanes under each indicated ORF contain the same sample, the second lane containing a three-fold dilution of the sample in the first lane. The blot was probed with an anti-CI antibody. (B) Western blot of the same bacterial cultures as in (A) but grown for 1-hour post IPTG induction. The band above the  $\lambda$ CI-CsgB protein band is an unspecific band always seen when the CI antibody is used and the bands below the full-length  $\lambda$ CI-CsgB protein band represent degradation products. The signal seen at the top of the gel represents aggregated  $\lambda$ CI-CsgB protein stuck on the well.



## DISCUSSION

In carrying out the work presented in this chapter, we set out to identify cellular factors that could affect prion formation in the bacterial cytoplasm. We used the conversion of a cytoplasmic version of the curli component, CsgB, to the amyloid conformation as a surrogate for initial prion formation. Taking advantage of the observation that the toxicity of the  $\lambda$ CI-CsgB fusion protein correlates with the ability of the CsgB moiety to form amyloid-like aggregates, we designed a selection to identify factors that inhibit amyloid formation in *E. coli*. Using the ASKA overexpression library [27], we identified three ORFs that when overexpressed, seem to inhibit  $\lambda$ CI-CsgB amyloid dependent toxicity in *E. coli*. Importantly, these ORFs do not relieve the observed toxicity by merely reducing the levels of the  $\lambda$ CI-CsgB fusion protein in the *E. coli* cells. However, the mechanisms by which these ORFs relieve  $\lambda$ CI-CsgB mediated toxicity and potentially inhibit cytoplasmic amyloid formation remain to be investigated.

One of the identified ORFs encodes the conserved protein, YrhA, the function of which is not known. One of the other ORFs encodes the predicted protein, YdjO. Although not much is known about the function of this protein in the cell, studies in *E. coli* have shown that *ydjO* transcription is downregulated when cells have to utilize alanine or acetate as their carbon source [28]. Under these conditions, bacteria generally increase the number of expressed genes that are involved in metabolism and motility. Interestingly, in a temporal study of gene expression in *E. coli* biofilms, Domka *et al.* reported *ydjO* upregulation in biofilms following 7 hours of culturing [29]. Together, these reports suggest a role for YdjO in biofilm formation as it is transcriptionally

upregulated in biofilm conditions [29], and conversely, downregulated under conditions that stimulate high motility [28].

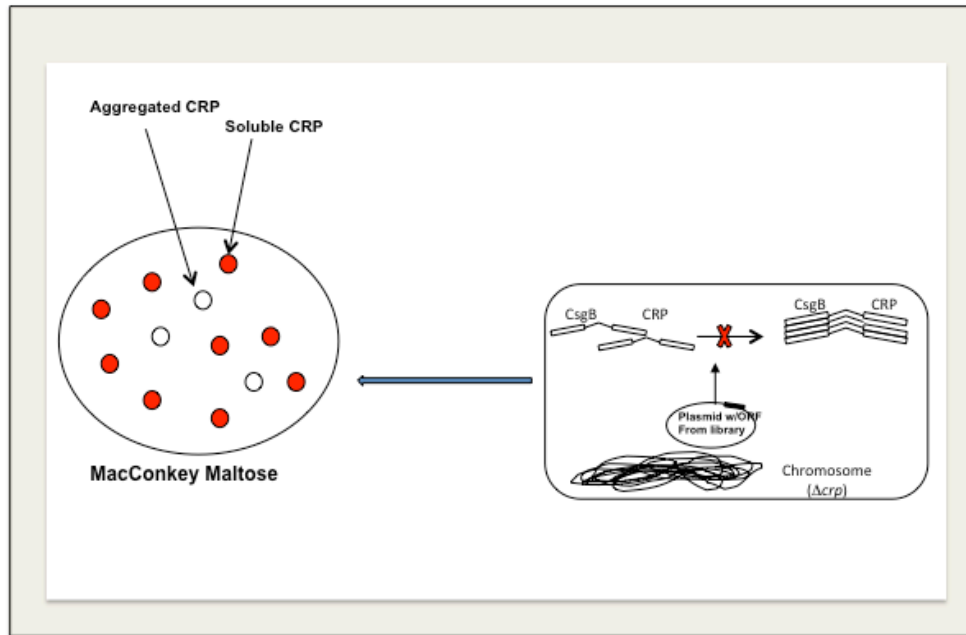
The third identified ORF encodes the cytoplasmic hydrolase MhpC (2-hydroxy-6-oxononatrienedioate hydrolase) involved in the catabolism of 3-(3-hydroxyphenyl) propionate, a carbon source used by bacteria in the absence of other preferred carbon sources [30]. A report has recently shown that *mhpC* is upregulated in *E. coli* cells deleted for *rpoS*, the gene encoding the  $\sigma^S$  subunit of the RNA polymerase, which controls the expression of genes involved in general stress resistance [31]. Interestingly, this same report indicated that RpoS contributes to biofilm maturation by regulating many genes involved in energy metabolism, motility and stress response. Cells deleted for *rpoS* cannot establish mature biofilms and it is in these cells that the group reports upregulation of the *mhpC* gene. Providing more support for the negative correlation between MhpC production and biofilm formation, the group reported *mhpC* downregulation in wild-type biofilms [31].

Although these reports do not suggest an explanation for how the identified ORFs might influence cytoplasmic amyloid formation, it is intriguing that they link YdjO and MhpC specifically to biofilm formation (though the correlations go in opposite directions). Furthermore, we cannot exclude the possibility that the ORF-encoded proteins influence, directly or indirectly, the ability of the  $\lambda$ CI-CsgB fusion protein to associate with the DNA and thereby relieve fusion protein toxicity.

A crucial question is whether these ORF-encoded proteins indeed lower the aggregating ability of the  $\lambda$ CI -CsgB fusion protein. In principle, we could address this question by using the standard SDD-AGE method (described in CHAPTER 2) to

visualize SDS-stable fusion protein aggregates in cells co-expressing one of the selected ORFs or a control ORF. Unfortunately, however, the current system does not allow us to address the question in this way; whereas we can grow cells expressing the selected ORFs and the  $\lambda$ CI-CsgB fusion protein and analyze their extracts for aggregates, we cannot do the same with the cells expressing a control ORF, as the aggregation of the  $\lambda$ CI-CsgB fusion protein inhibits the growth of such cells.

To test the effects of these ORFs on CsgB aggregation, we can instead take advantage of a previously characterized fusion protein consisting of the CsgB moiety at the N-terminus of the global transcription activator CRP. In this context, conversion of CsgB to the amyloid fold does not result in cell toxicity (S. J. Garrity, unpublished data). Previous findings indicate that aggregation of the CsgB-CRP fusion protein leads to sequestration of the CRP protein and therefore result in a  $\text{Crp}^-$  phenotype, which can be visualized as pale colonies on MacConkey maltose indicator plates (S. J. Garrity, unpublished data). (Note that CRP positively regulates genes required for the utilization of maltose as a carbon source.) Specifically, the CsgB-CRP fusion protein would be introduced alongside the mobilizable plasmid carrying one of the selected ORFs or a control ORF into a strain that has been deleted for the *crp* gene. If the ORF-encoded protein present on the mobilizable plasmid reduces the aggregating ability of the CsgB-CRP fusion protein, the increase in the amount of soluble CRP in the cell should result in a  $\text{Crp}^+$  phenotype and the cells should form red (or pink) colonies on MacConkey maltose indicator medium (Figure 3.5). In contrast, cells containing a control ORF will form pale colonies when grown under the same conditions.



**Figure 3.5: Experimental design to determine whether the selected ORFs decrease formation of CsgB amyloids.** Aggregation of the CsgB-CRP fusion protein results in a Crp<sup>-</sup> phenotype, which is visualized as pale colonies on indicator medium. Overexpression of an ORF that inhibits amyloid formation, will result in a Crp<sup>+</sup> phenotype, visualized as red colonies indicator plates.

Work in yeast has clearly demonstrated the critical importance of the cellular chaperone machinery in the formation and propagation of yeast prions, but aspects of these interactions remain unclear. Published work from this lab, as well as the work presented in CHAPTER 2, has established *E. coli* as a useful system for studying the behavior of yeast prions [18]. In addition, work from a former graduate student in the lab, Dr. Sean Garrity, has shown that altering chaperone levels influences the aggregation state of the Sup35 in *E. coli*. For instance, overproduction of ClpB, the Hsp104 homolog, not only seems to reduce the average size of the Sup35 polymers, but also seems to increase the infectivity of such aggregates. In addition, overproduction of the DnaK system in *E. coli*, composed of DnaK (Hsp70), DnaJ (Hsp40) and GrpE (nucleotide exchange factor), alters the fluorescence pattern of the Sup35 protein from big twisted ribbons (correlated with amyloid structures) to primarily a diffuse pattern. The fluorescence pattern of New1, another yeast prion protein that can access the amyloid fold in bacteria (see CHAPTER 2), is also altered under these conditions from distinct foci (indicative of aggregation) to diffuse fluorescence. Although further studies are required to investigate the interaction of the DnaK system and prion amyloids in *E. coli*, these results suggest that alterations in the balance of bacterial chaperones can influence behavior of yeast prion proteins.

Based on this preliminary work, it would be interesting to test the effects of the factors identified in this chapter (pending the results of experiments performed with the CsgB-CRP fusion protein) on the abilities of the New1 and NM proteins to access the prion conformation. In addition, one could also investigate the effects of the factors identified here on the propagation of the NM prion in the *E. coli* cytoplasm (see

CHAPTER 2). Collectively, these experiments will help identify the cellular players involved in amyloid formation and maintenance in the *E. coli* cytoplasm.

## **ACKNOWLEDGEMENTS**

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## **MATERIALS AND METHODS**

### **Plasmids, Strains and Cell growth**

Bacterial cultures were grown overnight and then diluted to OD<sub>600</sub> 0.02 in LB supplemented with the appropriate antibiotics (Carbenicillin 100 µg/ml; Chloramphenicol 25 µg/ml) grown for about 3 hours at 37°C and induced with the appropriate inducers IPTG at 125µM) for either 30minutes or 1hour.

For the plate selection experiment, bacterial cultures cotransformed with the ASKA plasmid and the λCI -CsgB construct were plated on LB plates supplemented with the appropriate antibiotic and IPTG. The plates were incubated overnight at 37°C.

The λCI -CsgB construct was produced from pACYC-derived plasmids under the control of the IPTG inducible promoter lacUV5.

### **Bacterial Extract Preparation**

25ml cultures were grown to the indicated times and OD<sub>600</sub> the was recorded. Bacterial cells were pelleted by centrifugation at 3000g for a period of 10 minutes. The bacterial pellets were then resuspended in 90% formic acid to solubilize the λCI -CsgB aggregates. The formic acid was removed by spinning the samples in a vacuum concentrator for about 10 minutes. The pellets were then resuspended in 1X SDS-PAGE loading dye and boiled for 15 minutes.



**Western blot**

Blots were probed with anti-CI (gift from Dr. Jon Beckwith). Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling ab7074). Blots were detected using ECLplus Western Blotting Detection System (GE Healthcare).

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## **CHAPTER 4**

### **Discussion**

## SUMMARY

The term prion was first coined by Prusiner in 1982 to describe the proteinaceous infectious particle causative of scrapie in sheep [1, 2]. Now it refers to self-propagating protein aggregates, which are usually comprised of highly structured beta-sheet-rich fibrils known as amyloids [3, 4]. The discovery of their existence in yeast has dramatically improved our understanding of prions, although many questions still remain unanswered. Yeast prions do not generally cause cell death and in fact act as heritable protein based genetic elements [5]. They confer new and stably inherited phenotypic states on the yeast cells harboring them.

Studies in yeast have suggested a role for molecular chaperones in prion behavior. The most thoroughly characterized example is the Hsp104 chaperone, which plays a critical role in prion propagation. It is believed that Hsp104 cleaves large prion aggregates into smaller, more easily propagated species. However, it is still not clear whether Hsp104 works alone or with other chaperones in this function. It is also not known if the Hsp104 generated propagons are passaged to daughter cells in an active or passive process [6, 7]. Interestingly, no Hsp104 homolog has been found in humans, although one exists in plants. It should be noted that other chaperones have also been implicated in both the *de novo* biogenesis of prions and in their propagation, but their effects are complex and have been difficult to tease apart.

Since the discovery of yeast prions many years ago, the number of known and putative yeast prions has grown enormously and continues to grow. However, it is not known if prions exist outside the eukaryotic domain of life. Bacteria produce extracellular non-prion amyloids, but it is not known if bacteria contain proteins that can

access a prion-like state. If such proteins exist, bacteria could utilize prion-like aggregates as epigenetic switches that could potentially help the cells adapt to a variety of stress conditions.

As a first step in addressing the existence of bacterial prions, it is important to investigate the ability of the bacterial cytoplasm in supporting the *de novo* biogenesis and propagation of prions. Using a well-characterized *Saccharomyces cerevisiae* prion protein, Sup35, a study from this lab already established that infectious protein aggregates could indeed form in bacterial cells. The study also demonstrated that the formation of these aggregates was dependent on the presence of amyloid aggregates of another yeast prion protein, New1. The dependence of Sup35 aggregation on the presence of another aggregated prion protein is also observed in yeast, where it is known as the PIN effect. It is interesting that the PIN dependence of Sup35 aggregation in yeast was recapitulated in bacteria, although bacteria diverged from eukaryotes more than 2 billion years ago.

In the work presented in this dissertation we examined whether the infectious Sup35 aggregates formed in the bacterial cytoplasm in the presence of PIN could be propagated in bacteria. We found that, once formed, the Sup35 aggregates could be maintained in the bacterial cytoplasm for about 90 generations (CHAPTER 2). Propagation of these aggregates was not dependent on the presence of the transplanted PIN factor and this observation recapitulated the *in vivo* behavior of the Sup35 prion in yeast (namely, PIN is required for the *de novo* formation of the Sup35 prion, but not for its maintenance). Our experimental design tested for maintenance for up to about 90 generations and although we have no evidence to suggest that aggregate maintenance would not continue past this

point, additional experiments need to be carried out to answer the question of maintenance past 90 generations.

We also sought to identify bacterial cellular factors that could affect amyloid aggregation in the bacterial cytoplasm (CHAPTER 3). We developed a selection capable of detecting factors that could interfere with amyloid formation in the *E. coli* environment. We used the aggregation of a well-studied bacterial protein, CsgB, as a surrogate for the initial conversion of a prion protein to the amyloid/prion conformation. In designing our selection, we also relied on the observation that, when fused to the bacteriophage lambda CI protein, CsgB causes cell toxicity that is dependent on the conversion of the fusion protein to the aggregated state. We used relief of toxicity as a readout in our selection to identify cellular factors that, when overproduced, could lower the aggregation propensity of the  $\lambda$ CI-CsgB fusion protein. We were able to identify three open reading frames (ORFs) that, upon overexpression, potentially relieve the  $\lambda$ CI-CsgB dependent toxicity. Although the mechanisms by which the ORF-encoded proteins relieve  $\lambda$ CI-CsgB mediated toxicity and potentially influence amyloid formation remain to be investigated, these proteins do not seem to relieve toxicity by merely downregulating fusion protein production or destabilizing the fusion protein.

The work presented in this dissertation demonstrates that *E. coli* is an experimental system useful in studying the behavior of known and putative prion proteins. It established that the bacterial cytoplasm is capable of supporting not only formation of prion aggregates, but also their propagation. It also identified three bacterial proteins as candidate factors that may modify the behavior of amyloid forming proteins in the bacterial cytoplasm. Together, these findings help establish a framework for using



bacteria to study the complex interaction of chaperones and prions and they increase our interest in the search for bacterial proteins that exhibit prion-like behavior.

## **FUTURE DIRECTIONS**

### **Experiments to better understand heritability of yeast prions in bacteria**

As previously mentioned, once formed, prions are stably inherited in the yeast cells harboring them. Although the work presented in this dissertation has shown that it is possible for yeast prions to be maintained in the bacterial cytoplasm, we have not yet been able to address the question of prion stability in bacteria. Two main issues with our current system hamper our ability to do so.

First, our system for studying heritability of the Sup35<sup>NM</sup> (hereafter referred to as NM) prion depends on the ability to remove the PIN-encoding gene, *new1*, from the system once the prion aggregates have formed. At the present time, *new1* is inserted in the bacteria chromosome at the lambda attachment site, and the excisionase machinery is provided on a plasmid under the control of a temperature sensitive promoter. This system results in low *new1* removal efficiency (CHAPTER 2), limiting the number of colonies available to us for the subsequent analysis of NM aggregate maintenance. A way to increase the efficiency of *new1* loss from the system would be to provide the *new1* gene on a plasmid that contains a temperature sensitive origin of replication. In this system, transition of the bacterial cells to the non-permissive temperature following initial NM aggregation would allow for easy removal of the PIN factor. We could then study the heritability of NM prions in bacterial clones that have lost the PIN factor.

Second, for reasons we do not fully understand, the system we use for the production of the NM protein results in highly variable protein levels, not only from experiment to experiment, but also from colony to colony in the same experiment as well as over time in the same clone. This makes the study of heritability especially difficult because the propagation of NM aggregates necessarily depends on the continuing synthesis of the NM protein. Although a range of NM protein levels can result in NM aggregation, it is expected that aggregates will no longer form once NM levels drop below a certain (unknown) threshold.

Upon initial consideration, it might seem possible to investigate the stability of the NM prion in our current system by simply looking for NM aggregates among bacterial clones with high levels of NM protein. The identification of clones that lack NM aggregates but contain NM levels similar to other NM aggregate-containing clones would suggest that NM aggregates are lost at a significantly higher frequency in bacteria than in the native yeast system. However, NM protein levels can vary during the course of growth of the bacterial culture (see CHAPTER 2). High protein levels at a certain point during growth provide no guarantee that those protein levels were consistently present in the bacterial culture. Since we cannot interpret the absence of NM aggregates even when we observe high NM protein levels at the time the cells are harvested, we cannot currently draw any conclusions concerning the stability of the NM prion aggregates in bacteria.

In order to study stability of the NM prion in bacteria we need to modify the current system so that NM protein levels remain constant throughout the experiment. Based on our initial studies, the fluctuation in the NM levels seems to be tied to the arabinose

inducible system driving expression of the NM encoding gene in bacteria. Specifically, we observe fluctuation in protein levels in the case of the NM protein, where expression of the gene is driven by an arabinose inducible promoter, but not in the case of the New1 protein, where expression of the gene is under the control of an IPTG inducible promoter (see SUPPLEMENT TO CHAPTER 2). Based on this observation, we suggest providing NM under the control of an IPTG inducible promoter. We expect that this new induction system would provide the consistent levels of NM needed for the study of prion stability in bacteria. This modification, combined with the new method of removing PIN from the system, would allow us to conduct a more quantitative analysis of prion stability in the bacterial cytoplasm. Current efforts in our lab are directed at implementing these changes in our experimental system.

In this new system, we would initially grow the starter culture under inducing conditions for both NM and New1. Since production of both proteins would now be dependent on IPTG, it would no longer be possible induce the synthesis of NM independently of New1 in the starter culture serving as the negative control (culture B; see CHAPTER 2). Instead, culture B would contain the plasmid providing NM alongside the corresponding empty plasmid used to provide New1. This would ensure that both the NM and New1 proteins are produced upon induction with IPTG in culture A, but only the NM protein is produced in culture B. Following formation of the NM aggregates in culture A, the bacterial cells would be transitioned to the non-permissive temperature for the temperature-sensitive replicon of the New1 plasmid. This would result in loss of the New1 encoding plasmid from culture A, and loss of the corresponding empty plasmid from culture B. We would then analyze extracts from bacterial cultures grown from a

number (e.g., 10) of individual colonies confirmed to have lost the *newI* gene; specifically we would assess NM protein levels by Western blot and test for the presence of aggregates by SDD-AGE. This same analysis would be carried out for both culture A and B derivatives. The fraction of NM aggregate-containing colonies among the number of culture A derived colonies analyzed in this round (Round 1 in Fig. 2.7 of CHAPTER 2) would provide information on the stability of the NM aggregates in the bacterial cytoplasm. For example, the presence of NM aggregates in all the colonies analyzed, would suggest that NM aggregates are stably maintained in *E. coli*. However, the presence of NM aggregates in only a portion of the analyzed colonies, would suggest that NM aggregates are not as stable in bacteria as they are in yeast. Following analysis in Round 1, we would pick a fraction of the NM-aggregate containing colonies (e.g., 4) and restreak them for single colonies for Round 2 (Fig. 2.7 of CHAPTER 2). For each of the 4 clones selected in Round 1, we would analyze extracts from overnight cultures grown from 10 individual colonies from Round 2 for NM protein levels and NM aggregates. This would allow us to calculate the frequency of NM-aggregate containing colonies in Round 2 for each of the 4 clones selected from Round 1. We would then proceed in the same way through the remaining rounds of Fig. 2.7, calculating the frequency of aggregate-containing colonies in each round for each of the 4 clones selected from Round 1. The same analysis would be carried out with culture B derivative clones from Round 1. This experiment would allow us to quantitatively study the stability of NM aggregates through all of the 4 rounds of Fig. 2.7. If the proportion of NM aggregate containing colonies does not change for each of the clones from round to round, we would conclude that the stability of the NM aggregates in the *E. coli* cytoplasm does not change upon

additional rounds of restreaking. If however, we observe a decrease in the proportion of NM-aggregate colonies as we move from round to round, we would conclude that NM aggregates cannot be maintained indefinitely in the bacterial cytoplasm and would eventually be lost following additional rounds of restreaking.

In addition to expanding our understanding of yeast prion heritability in bacteria, the redesigned system outlined above would allow us to test the effect of both bacterial chaperones and transplanted yeast chaperones on the processes of prion formation and propagation. In fact, preliminary work carried out by Dr. Sean Garrity, a former graduate student in the lab, already suggest a role for ClpB, the bacterial Hsp104 homolog, in yeast prion behavior in bacteria. Specifically, he showed that overproduction of ClpB not only decreased the average size of the NM aggregates, but it also increased their infectivity (S. J. Garrity, unpublished data).

Additionally, his preliminary experiments showed that overproduction of the *E. coli* DnaK/DnaJ chaperone system (the bacterial Hsp70/Hsp40 homolog) alters the fluorescence pattern of the NM-YFP fusion protein. When produced in the presence of the New1-CFP fusion protein, which serves as a PIN factor, the NM-YFP fusion protein forms twisted ribbon-like structures that transverse the length of the cell as analyzed by fluorescence microscopy [8]. In these same cells, the New1-CFP fusion protein forms a bright focus, from which the NM-YFP ribbons seem to emanate [8]. The presence of these NM-YFP ribbons correlates with SDS-stable NM aggregates as assayed by SDD-AGE. It is important to note that neither NM-YFP ribbons nor NM-YFP stable aggregates are observed in cells missing the PIN factor [8]. Interestingly, when S. J. Garrity overproduced the DnaK system, consisting of DnaK (Hsp70), DnaJ (Hsp40), and GrpE (a

nucleotide exchange factor) in *E. coli* cells producing both NM-YFP and New1-CFP, he observed that the fluorescence pattern of the NM-YFP protein changed from twisted ribbons to a primarily diffuse pattern throughout the cell, suggesting that the *E. coli* DnaK/DnaJ system might interact with prion aggregates. Similarly, the fluorescence pattern of the New1-CFP protein changed from distinct foci to a primarily diffuse pattern (S. J. Garrity, unpublished data).

Based on these observations, it would be interesting to test the effect of overproducing specific chaperones, bacterial or yeast, on the formation and infectivity of the NM prion, as well as on its stability in the bacterial cytoplasm. To test the effect of chaperone overproduction on the formation of NM aggregates, we would simply overproduce the specific chaperone in cells producing both NM-YFP and New1-CFP and analyze the bacterial extracts for NM-YFP protein levels and aggregates as compared to extracts from cells producing the same fusion proteins in the context of normal chaperone levels. The infectivity of the material generated under the two conditions (chaperone overproduction and normal chaperone levels) would then be tested by transforming yeast cells as previously described in CHAPTER 2. Additionally, we can test the effect of chaperone overproduction on other yeast proteins that form prion-like aggregates in the bacterial cytoplasm. The New1-CFP fusion protein would be a good candidate for this study as this protein has been shown to form SDS-stable aggregates in *E. coli* [8], though in this case there is no convenient assay for infectivity.

Testing the effect of chaperone overproduction on prion maintenance, as opposed to prion formation, would require additional tools. In particular, in the case of NM, we would need to place the specific chaperone(s) being studied under the control of a tightly

regulated promoter, enabling us to overproduce the chaperone only after initial formation of the NM aggregates. For these experiments, the NM aggregates would be allowed to form in cells producing the PIN factor and normal levels of the chaperone being studied. Then, bacterial cells would then be transitioned to conditions allowing for the simultaneous removal of the PIN factor and the overproduction of the chaperone. This set up would ensure that we test the effect of overproducing the chaperone only on the propagation of the NM prion, not on its formation. The cells would then be carried through the steps outlined in Figure 2.7, CHAPTER 2 and the bacterial extracts would be studied for presence of NM aggregates as previously described. The infectivity of these aggregates would also be analyzed. Important for the interpretation of the results of these experiments would be the results from parallel experiments where the PIN factor is still removed, but the chaperone is not overproduced following NM aggregation.

A promising area for immediate application of the system described here is the question of whether Hsp104 functions alone in prion propagation or whether it collaborates with the Hsp70/Hsp40 chaperone system the way it does in disaggregating and reactivating misfolded proteins after heat shock. *In vitro* studies have shown that Hsp104 can function alone in fragmenting prion fibers, however other *in vitro* studies suggest that this activity is enhanced by Hsp70/Hsp40 factors [9-11]. Moreover, studies suggest a role for the Hsp70/Hsp40 factors in prion propagation *in vivo* [12-15]. However, it is unclear whether or not Hsp104 can function independently of the Hsp70/Hsp40 chaperone system to facilitate prion propagation *in vivo*. Because studies of Hsp104/ClpB-mediated thermotolerance have shown that these factors co-operate with their respective Hsp70/Hsp40 partners in a species-specific manner, our modified

bacterial system would allow us to test the effect of Hsp104 on NM prion propagation in a cellular setting that lacks the cognate Hsp70/Hsp40 partners. In particular, prior studies suggest that the bacterially produced Hsp104 will not collaborate with the bacterial Hsp70/Hsp40 (DnaK/DnaJ) system [15-18]. The proposed experiment would be carried out in a *ΔclpB* strain in order to clearly distinguish between effects of Hsp104 and effects of ClpB (and also to avoid complications due to the possible formation of mixed ClpB/Hsp104 oligomers). Thus, we would first need to test the effect of deleting *clpB* (or depleting ClpB) on NM prion behavior (see below) before being able to interpret an experiment designed to test the effect of Hsp104. If we find that formation and/or propagation of the NM prion in the bacterial system depends on ClpB, then asking whether or not Hsp104 can substitute functionally for ClpB would be of particular interest.

The chaperone overproduction studies proposed here would be nicely complemented by deletion studies. Of course, the deletion studies would only be carried out with bacterial chaperones. To study the effect of the deletion of certain chaperones on NM prion formation in the bacterial cytoplasm, we would carry out experiments similar to the overproduction experiments with the modification that the specific chaperone would be deleted, instead of overproduced, and the behavior of the yeast prion in the deletion strains would be compared to the behavior in the strain producing normal levels of the chaperone. It is important to note that in the case of essential proteins, such as Hsp70 (DnaK), these deletion studies would have to be carried out in specific bacterial strains containing suppressor mutations. Studying the effect of chaperone deletion on NM prion maintenance would be straightforward only for chaperones that are not required for



formation of the NM prion. For chaperones that are required for NM prion formation, a depletion strategy could be used to assess whether or not the same chaperone is required to maintain the prion, but the experimental design would be challenging given the need to remove the PIN factor and simultaneously remove or deplete the chaperone.

Finally, our modified bacterial system could be used to study the heritability of other known yeast prions as well as study their interaction with the chaperone systems. The approach to studying the heritability of yeast prions that depend on PIN for initial conversion to the prion fold would be similar to the modified approach we plan to use to study the heritability of the NM prion. On the other hand, in order to study the heritability of yeast prions that do not depend on a PIN factor for their initial formation, we would need to be able to identify protein levels that suffice for prion maintenance but not for initial formation. This would require a vector that provides for a well controlled range of protein concentrations as a function of inducer concentration. To carry out the experiment, we would allow the prion to form by culturing the cells at an induction level experimentally shown to allow for initial aggregate formation. We would then transition the cells to lower induction levels shown to be insufficient for *de novo* aggregate formation. This set-up would allow us to study the propagation of these prions in *E. coli*. Additionally, we could test the effect of chaperones on their behavior in the same manner suggested for the NM prion.

### **Further characterization of the three ORFs implicated in relieving the toxicity of the CI-CsgB fusion protein**

In the work presented in this thesis, we identified three bacterial open reading frames (ORFs) that potentially inhibit cytoplasmic amyloid aggregation. However, with our

current set-up, we are unable to test their effect on the formation of SDS-stable aggregates due to the fact that the CI-CsgB fusion protein is toxic (except under conditions where one of the identified ORFs is being overexpressed). To circumvent this problem, the effect of the overexpression of the ORF on amyloid aggregation can be tested under conditions where the CsgB aggregation in the bacterial cytoplasm does not correlate with cell toxicity.

Previous studies in the lab have shown that when CsgB is fused to the global transcription activator CRP, the resulting fusion protein can still access the amyloid conformation. Importantly, in this context, fusion protein aggregation does not cause cell toxicity. Additionally, *Δcrp* cells containing the CsgB-CRP fusion protein exhibit a *Crp*<sup>-</sup> phenotype (visualized as pale colonies on MacConkey maltose indicator plates), presumably because the CRP moiety is sequestered in fusion protein aggregates and unable to activate transcription. In fact, the aggregation deficient version of the CsgB protein, CsgB Q117R, described in CHAPTER 3, was identified by introducing random mutations into the *csgB* moiety of the *csgB-crp* fusion gene, transforming the mutagenized library into a *Δcrp* reporter strain, and screening for red colonies on MacConkey maltose indicator plates (S. J. Garritty, unpublished data).

The use of the CsgB-CRP fusion protein will allow us to test directly whether or not overexpression of the identified ORFs influences the aggregation propensity of the CsgB moiety. A *Δcrp* reporter strain would be co-transformed with the CsgB-CRP encoding plasmid and one of the identified ORFs (or a control ORF). Transformants would then be cultured under inducing conditions for both the CsgB-CRP and ORF constructs, and cell extracts would be analyzed for SDS-stable fusion protein aggregates by SDD-AGE. In

parallel, we would analyze the cell extracts for fusion protein levels by Western blot to ensure that the experimental samples and the control sample contain similar amounts of fusion protein. If the ORF present reduces the aggregating ability of the CsgB-CRP fusion protein, it would result in a weaker or absent SDD-AGE smear as compared to the sample containing the control ORF. Additionally, such an ORF would result in red or pinker colonies (when grown on MacConkey indicator medium supplemented with inducers for both constructs) as compared to the pale colonies formed by cells containing CsgB-CRP and a control ORF, providing us with another method to investigate the aggregation state of the CsgB-CRP fusion protein.

The overexpression experiments would be complemented nicely by deletion experiments, where we would test the effect of deleting the ORFs on the aggregating propensity of the CsgB-CRP fusion protein. If ORF overexpression inhibits the aggregation of the CsgB-CRP fusion protein, we would expect their deletion to either have no effect on the aggregation of the fusion protein or result in an increased aggregation of the CsgB-CRP fusion protein as compared to conditions of control ORF overexpression. For these studies, we would initially test the effect of deleting each of the three ORFs, one at a time, on the aggregating propensity of CsgB-CRP. These experiments would be carried out similarly to the overexpression experiments, except that we would compare the levels of aggregated CsgB-CRP fusion protein in cells lacking one of the ORFs to those in the wild-type strain. Although very unlikely, it is possible to imagine that all of the identified ORFs could inhibit CsgB aggregation by the same mechanism, and could therefore compensate for each other's absence. In that scenario, deleting only one of the ORFs would not have any effect on the behavior of the CsgB-

CRP fusion protein and deleting all of the ORFs at the same time would be required to study their effect on the aggregation of the CsgB-CRP fusion protein.

If the above experiments demonstrate that the ORFs reduce the aggregation propensity of the CsgB-CRP fusion protein, it would be interesting to investigate whether they also inhibit conversion to the amyloid fold for other amyloid forming proteins, in addition to CsgB-CRP. As a first step in addressing this question, we can easily test the effect of their overexpression on the formation of NM and New1 aggregates in the bacterial cytoplasm. If overexpression of any of these ORFs interferes with a critical step in amyloid formation, we would expect to see decreased amounts of aggregated NM and New1 (as assessed by SDS-AGE) when these aggregates are allowed to form in cells overexpressing that ORF. These experiments would be carried out similarly to experiments designed to test the effect of chaperone overproduction on aggregate formation.

Finally, the newly designed system for the study of prion heritability in *E. coli* could be used to investigate a possible effect of the overexpression of these ORFs on prion heritability. The set-up for these experiments would be very similar to the one described to test the effect of chaperone overproduction on NM prion stability. Combined with the other experiments described here, these studies would help us better understand the interaction of bacterial cellular factors with yeast prions and amyloidogenic proteins.

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